METABOLISM OF SECOND-GENERATION ANTIPSYCHOTICS BY URIDINE DIPHOSPHATE-GLUCURONOSYLTRANSFERASES

A Dissertation in
Cell and Molecular Biology
by
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ABSTRACT

Second-generation antipsychotics (SGA) are a widely prescribed class of drugs (1, 2) for the management of many disorders including schizophrenia, bipolar disorder, and treatment-resistant depression. The number of SGAs on the pharmaceutical market and their popularity has increased as clinical trials showed higher rates of responders, better functional capacity, an improved quality of life, and fewer extrapyramidal symptoms (EPS) and prolactin elevation side effects compared to first-generation antipsychotics (FGA). SGAs are recommended as the first-line treatment for schizophrenia and treatment resistant depression (3, 4).

Nevertheless, SGAs are associated with numerous adverse effects, most notably marked weight gain leading to obesity, metabolic syndrome, type II diabetes, dyslipidemia, and heart disease (5). Compared to the general population, SGA adverse effects shorten patient life-span by an estimated 25 years (6) primarily due to cardiovascular disease (CVD), increased medication non-adherence and clinical burden, costing billions of dollars in healthcare annually (7-9). The most severe weight gain phenotypes occur with the SGAs clozapine (CLZ) and olanzapine (OLZ). Thus, it is important to identify mechanisms that underlie SGA adverse effects and develop interventions to prevent SGA-associated weight gain.

Pharmacogenetics is the study of how genetic variation affects pharmacokinetics and pharmacodynamics, thereby influencing the efficacy and tolerability of a drug (10-12). The goal of pharmacogenetic studies is to identify genetic targets that predict the most efficacious drug and dose for a given individual, or reveal an individual’s risk of treatment failure or adverse effects before starting a specific treatment, thus providing “personalized medicine.” SGA pharmacogenetic studies and individualized medicine are vital to improving outcomes using currently available SGAs and to designing more efficacious treatments in psychiatric medicine. Treatment effectiveness trials show that most SGAs work in a subset of patients and, in other
patients, either lack efficacy or cause debilitating metabolic adverse effects that discourage patient adherence to treatment (13-16). It is not clear which pharmacogenetic targets, if any, predispose an individual to treatment efficacy or failure with SGAs. However, plasma concentrations of SGAs reflect brain concentrations of the drugs and have been correlated to SGA response, risk of weight gain and other adverse effects (17-22). Interindividual plasma concentrations are not necessarily predicted by the dose administered (17), suggesting that genetic variability in genes coding for drug metabolizing enzymes (DMEs) could affect SGA efficacy and tolerability (19).

DMEs are classified as Phase I or Phase II depending on the type of reaction they catalyze. The uridine diphosphate (UDP)-glucuronosyltransferases (UGTs) are one group of Phase II enzymes that mediate greater than one-third of Phase II drug metabolism (23) and influence the plasma levels and clearance of a given compound (24). UGTs are important for the biotransformation and clearance of many SGAs as evidenced by plasma and urine metabolites in humans (25-27). CLZ undergoes extensive biotransformation; 97% of the administered dose is metabolized (17) and seven CLZ glucuronides have been identified in humans (26-28). One of these glucuronides is of the abundant, active metabolite N-desmethylclozapine (dmCLZ) (28) that is involved in causing agranulocytosis in 1-2% of patients taking CLZ (29); glucuronidation may be an important clearance mechanism for the reactive metabolite. CLZ plasma levels vary widely among patients administered the same dose (3-1507 ng/ml (17, 30, 31) and therapeutic response is related to plasma concentrations rather than drug dose (17). UGT activity against CLZ has been partially characterized: In vitro, UGT1A4 forms tertiary and quaternary CLZ-glucuronide metabolites, and the UGT1A4L48V polymorphism was twice as efficient as wild-type UGT1A4 (32). This genetic difference, along with the extensive role that UGTs play in the metabolism and clearance of CLZ (17, 19, 26), suggests that UGT pharmacogenetic factors influence CLZ plasma levels. But, for CLZ and many SGAs, the UGTs that are enzymatically active have either not
been characterized or only partially characterized. Additionally, the effects of UGT polymorphisms are largely unknown \textit{in vitro} or \textit{in vivo}.

Glucuronide metabolic products account for 25\% of the administered OLZ dose, making it the major OLZ metabolic pathway. The three most abundant OLZ-glucuronide metabolites in humans are: OLZ-10-\textit{N}-glucuronide isomer 1, OLZ-10-\textit{N}-glucuronide isomer 2, and OLZ-4’-\textit{N}-glucuronide. Previous \textit{in vitro} studies using baculovirus-infected insect cells demonstrated that UGT1A4 is active against OLZ, with no activity observed for UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B7. However, kinetic data for glucuronidation of OLZ could not be established for human liver microsomes (HLM) using OLZ concentrations spanning the apparent \(K_M\) of UGT1A4, suggesting that other untested UGT enzymes contribute to OLZ glucuronidation \textit{in vivo} (33). There is interindividual variability in OLZ clearance (20, 21, 34-37) and efficacy is positively correlated with plasma concentrations up to a threshold value (21). Weight gain and obesity are seen only in a subset of patients taking OLZ (38) and the evidence suggests that there is a dose-response relationship between OLZ serum concentrations and metabolic outcomes (39-42).

Studies performed for this dissertation characterized UGTs involved in the metabolism and clearance of the severe weight gain-inducing SGAs CLZ, dmCLZ, and OLZ. The first study investigated which UGT enzymes were active against CLZ, dmCLZ, and OLZ and characterized their kinetics. The UGT enzymes active against CLZ are UGTs 1A1, 1A3, 1A4, and 2B10; dmCLZ is UGT1A4; and OLZ are UGTs 1A4 and 2B10. In the second study, the kinetic profiles of these UGTs and their functional polymorphisms were established. Stably-transfected HEK293 cell lines that over-express each individual UGT were used in the second study and provided evidence of pharmacogenetic determinants of CLZ, dmCLZ, and OLZ metabolism \textit{in vitro}. The third study demonstrated that genetic variants in these UGTs alter glucuronidation activity and kinetics of human livers \textit{in vitro}. Finally, the fourth study investigated the effects of UGT genetic variants \textit{in vivo}, and their relationship to SGA efficacy and adverse effects.
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LIST OF ABBREVIATIONS

5-HT<sub>n</sub>  Serotonin<sub>(receptor subtype)</sub>
ABC          Adenosine Triphosphate-binding cassette
Ach          Acetylcholine
ADA          American Diabetes Association
AhR          Aryl hydrocarbon receptor
APA          American Psychiatric Association
BDNF         Brain-derived neurotrophic factor
BPRS         Brief psychiatric rating scale
cAMP         Cyclic adenosine monophosphate
CATIE         Clinical antipsychotic trials of intervention effectiveness
Cdx          Caudal-related homeodomain protein
CGT          UDP-galactose ceramide galactosyltransferase enzyme
CLZ          Clozapine
CV           Cardiovascular
CVD          Cardiovascular disease
CYP450        Cytochrome P450
D<sub>n</sub>     Dopamine<sub>(receptor subtype)</sub>
DA           Dopamine
DARPP        Dopamine- and cAMP-regulated phosphoprotein
dmCLZ        N-desmethylclozapine
DME          Drug metabolizing enzyme
DMEM         Dulbecco’s modified eagles medium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>eIF</td>
<td>Eukaryotic initiation factors</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>EPS</td>
<td>Extra pyramidal symptoms</td>
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<tr>
<td>FGA</td>
<td>First generation antipsychotic</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney 293 cells</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<tr>
<td>HLM</td>
<td>Human liver microsome</td>
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<tr>
<td>HNF</td>
<td>Hepatic nuclear factor</td>
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<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Receptor affinity</td>
</tr>
<tr>
<td>LTG</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple-drug resistance</td>
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<tr>
<td>MEPS</td>
<td>Medical expenditure panel survey</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OLZ</td>
<td>Olanzapine</td>
</tr>
<tr>
<td>PANSS</td>
<td>Positive and negative syndrome scale</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pbx</td>
<td>Pre-B cell homeobox transcription factor</td>
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<tr>
<td>PCP</td>
<td>Phencyclidine</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PGP</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PM</td>
<td>Poor metabolizer</td>
</tr>
<tr>
<td>QALYs</td>
<td>Quality-adjusted life-years</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SGA</td>
<td>Second generation antipsychotic</td>
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<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
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<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UM</td>
<td>Ultra metabolizer</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>(V_{max})</td>
<td>Maximum velocity</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
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This dissertation and series of studies represents a collaborative effort of many individuals without whom the collected body of work would not have been possible. I would thus like to acknowledge the contributions of the following people:

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My husband Samuel Ridout, for being my colleague, best friend, sage, humorist, and love of my life.
Chapter 1

INTRODUCTION

Background and Significance

Second-generation antipsychotics (SGA) are a widely prescribed class of drugs (1, 2) in psychiatric medicine for management of disorders including schizophrenia, bipolar disorder, and treatment-resistant depression. Since the introduction of clozapine (CLZ), the first SGA in the 1970s, the popularity and number of compounds in the class of SGA drugs have grown. SGA popularity increased as clinical trials reported higher rates of responders, better functional capacity, an improved quality of life, and fewer extrapyramidal symptoms (EPS) and prolactin elevation in patients taking SGAs compared to first-generation antipsychotics (FGA). Currently, the American Psychiatric Association (APA) recommends SGAs as the first-line treatment for schizophrenia and treatment-resistant depression (3, 4).

At the same time, SGAs are associated with their own myriad of adverse effects, most notably marked weight gain leading to obesity, metabolic syndrome, type II diabetes, dyslipidemia, and heart disease (5). SGA adverse effects shorten patient life-span by an estimated 25 years (6) primarily due to cardiovascular disease, compared to the general population, increase medication non-adherence and clinical burden, and amount to millions of dollars in healthcare costs annually (7-9). The most severe weight gain phenotypes occur with the SGAs CLZ and olanzapine (OLZ). Thus, it is important to identify mechanisms that underlie SGA adverse effects and develop interventions to prevent SGA-associated weight gain.

Pharmacogenetics is a term that was first coined nearly a half century ago to describe how inherited variation can influence responses to medications. Specifically, how genetic
variation influences drug efficacy and tolerability (10-12). Pharmacogenetic studies focus on how genetic variation governs pharmacokinetic measures, such as absorption, distribution, metabolism and excretion (ADME), and pharmacodynamic mediators, such as receptors, drug transporters, and downstream second messengers, both of which determine drug efficacy and tolerability. The goal of pharmacogenetic studies is to identify genetic variation that affects the pharmacokinetics and pharmacodynamics of a drug such that an individual taking that drug would be more or less likely to respond to treatment and experience adverse effects. Once these targets are identified, a simple genetic screening test could be used to identify patients with a specific genetic profile. This would allow prediction of the most efficacious drug and dose for a given individual, or reveal an individual’s risk of treatment failure or adverse effects before starting a specific treatment, thus providing “personalized medicine.” Additionally, pharmacogenetics may inform the rational development of safer and more efficacious treatments by elucidating the pathways by which drugs act to treat illness and provoke unwanted adverse effects.

SGA pharmacogenetic studies and individualized medicine are vital to improving treatment using currently available SGAs and to designing more efficacious treatments in psychiatric medicine. Treatment effectiveness trials show that most SGAs work in a subset of patients and, in other patients, either lack efficacy or cause debilitating metabolic adverse effects that discourage patient adherence to treatment (13-16). While a number of pharmacogenetic targets have been studied as mediators of pharmacokinetic and pharmacodynamic factors, it is not clear which predispose an individual to treatment efficacy or failure. Plasma concentrations of SGAs are a good measure of brain concentrations of the drugs and have been correlated to SGA response, risk of weight gain and other adverse effects (17-22). Optimal plasma concentration ranges have been defined for the severe weight gain-inducing SGAs that maximize efficacy and tolerability, however, plasma concentrations measured in an individual are not necessarily
predicted by the dose administered (17). Large interindividual differences in SGA plasma concentrations after the same administered dose are hallmarks of pharmacogenetic variability in drug metabolism implicating drug metabolizing enzymes (DMEs) as mediators of SGA efficacy and tolerability (19).

Drug metabolism is an important mediator of an individual’s pharmacokinetic profile, including plasma concentrations, clearance, and distribution (43) and is performed by a number of hepatic and extra-hepatic enzymes. In general, drug metabolism converts a lipophilic compound to a more readily excreted hydrophilic compound and can be involved in activation or deactivation of a chemical. The rate of drug metabolism is an important determinant of the duration and intensity of a drug’s pharmacological action, mediating pharmacokinetic parameters such as plasma concentrations, drug clearance and distribution (43) and pharmacogenetic variability in DMEs strongly determines this rate.

DMEs are classified as Phase I or Phase II depending on the type of reaction they catalyze. Phase I Enzymes characteristically add or unmask a polar group through an oxidation, reduction, hydrolysis, cyclization or decyclization reaction. Phase II enzymes catalyze conjugation reactions, usually interacting with a polar functional group. Both Phase I and II enzymes are responsible for formation of major active metabolites and for the inactivation and elimination of SGAs. The uridine diphosphate (UDP)-glucuronosyltransferases (UGTs), one group of Phase II enzymes, increase the solubility and clearance of a compound by catalyzing the addition of a glucuronic acid moiety and creating a water soluble drug-glucuronide.

UGT enzymes mediate greater than one-third of Phase II drug metabolism (23) acting both on the parent compound, forming a primary metabolite, or on Phase I metabolic products of the compound, forming secondary metabolites. Both primary and secondary glucuronide metabolites contribute to the pharmacokinetic variables of plasma concentrations and clearance.
of a given compound (24). UGT enzymes play an important role in interindividual response to drug therapy, as in the case of tamoxifen, irinotecan, or morphine, and should be considered when starting a dosed drug regime with a patient (44-46).

UGT enzymes are important for the biotransformation and clearance of many SGAs as evidenced by plasma and urine metabolites in humans (25-27). CLZ undergoes extensive biotransformation as only 3% of the administered dose is excreted unchanged (17) and seven CLZ-glucuronides have been identified in humans (26-28). Three of these metabolites account for approximately 30% of human CLZ metabolites (26); the rest have not yet been quantified (27). One of these glucuronides is of the abundant, active metabolite N-desmethylclozapine (dmCLZ) (28) that is involved in causing agranulocytosis in 1-2% of patients taking CLZ (29); glucuronidation may be an important clearance mechanism for the reactive metabolite. CLZ plasma levels vary widely among patients administered the same dose (10-974 ng/ml (17, 30)) and therapeutic response is related to plasma concentrations rather than drug dose (17). Interindividual variability in CLZ plasma concentration is explained in part by smoking status, age, body weight, and cytochrome (CYP) P450 enzyme activity (30, 47). However, these factors explain less than half of the variability in plasma levels and fail to explain why approximately 15% of patients cannot achieve the recommended therapeutic drug plasma range of 300-600ng/ml (48), which is a critical barrier in the treatment of psychiatric patients. Furthermore, these factors do not account for interindividual variation in plasma levels of dmCLZ (31), nor do levels of this metabolite correlate with CLZ dose (31). UGT enzyme activity against CLZ has been partially characterized: In vitro, UGT1A4 forms tertiary and quaternary CLZ-glucuronide metabolites, and the UGT1A4<sup>L48V</sup> polymorphism was twice as efficient as wild-type UGT1A4 (32). This, along with the extensive role that UGTs play in the metabolism and clearance of CLZ (17, 19, 26), suggests that UGT pharmacogenetic factors influence CLZ plasma levels. But, for CLZ and many SGAs, the UGTs that are enzymatically active have either not been characterized or only partially
characterized. Additionally, the effects of UGT polymorphisms are largely unknown in vitro or in vivo.

Glucuronidation is the major mode of OLZ metabolism, accounting for 25% of the administered dose. Three major glucuronide metabolites are made in humans: OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, and OLZ-4’-N-glucuronide. Previous in vitro studies using baculo-virus-infected insect cells demonstrated that UGT1A4 is active against OLZ, with no activity observed for UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B7. However, kinetic data for glucuronidation of OLZ could not be established for human liver microsomes (HLM) using OLZ concentrations spanning the apparent K_M of UGT1A4, suggesting that other untested UGT enzymes contribute to OLZ glucuronidation in vivo (33). There is interindividual variability in OLZ clearance (20, 21, 34-37) and studies indicate clinical outcomes are positively correlated with plasma concentrations up to a threshold value (21). Weight gain and obesity occur only in a subset of patients taking OLZ (38), and the evidence suggests that there is a dose-response relationship between OLZ serum concentrations and metabolic outcomes (39-42). Recently the UGT1A4 *3 allele was shown to be a significant predictor of OLZ plasma levels in vivo, resulting in a 5-fold reduction compared to the wild-type allele (36).

Thus, this series of dissertation studies characterized the UGT enzymes involved in the metabolism and clearance of the severe weight gain-inducing SGAs CLZ, dmCLZ, and OLZ, the kinetics of UGT enzyme reaction with these SGAs, and UGT-mediated pharmacogenetic determinants of SGA metabolism in vitro and in vivo. The first two studies were designed to characterize the UGT enzymes active against CLZ, dmCLZ, and OLZ and establish the kinetic profiles of these UGTs and their functional polymorphisms. Using stably-transfected HEK293 cell lines that over-express each individual UGT, these studies provided evidence of pharmacogenetic determinants of CLZ, dmCLZ, and OLZ metabolism in vitro. The third study investigated the hypothesis that genetic variants in these UGTs would alter glucuronidation
activity and kinetics of human livers studied *in vitro*. Finally, the fourth study investigated the effects of UGT genetic variants *in vivo*, and investigated their relationship with SGA efficacy and adverse effects.

**Specific Aims and Hypotheses**

**Specific Aim 1**

The purpose of the study "Screening and kinetics of UDP-glucuronosyltransferase enzymes with activity against severe weight gain-inducing second-generation antipsychotics,” was to confirm, in a human cell line, previous reports of UGT enzyme activity against SGAs to fully characterize which human UGTs have enzymatic activity against the severe weight gain-inducing SGAs CLZ, its active metabolite dmCLZ, and OLZ in a baculovirus-infected insect cell microsome model. Cellular homogenates were prepared from human cells over-expressing human UGTs.

**Hypothesis 1A:** UGTs with previously reported enzymatic activity against CLZ, its active metabolite dmCLZ, and OLZ in baculosome-virus-infected insect cell microsomes will demonstrate enzymatic activity against these SGAs in cellular homogenates of human cells overexpressing individual UGTs.

**Hypothesis 1B:** A complete screening of all 15 UGTs known to be involved in xenobiotic metabolism will reveal additional UGTs that are enzymatically active against CLZ, its active metabolite dmCLZ, and OLZ.
Specific Aim 2

The purpose of the study "Functional characterization of polymorphisms of UDP-glucuronosyltransferase enzymes responsible for the glucuronidation of severe weight gain-inducing second-generation antipsychotics,” was to characterize the kinetics of the enzymatic reactions catalyzed by reported functional variants of UGTs shown, in Specific Aim 1, to have enzymatic activity against the severe weight gain-inducing SGAs CLZ, its active metabolite dmCLZ, and OLZ.

Hypothesis 2: Functional variants of UGT enzymes with activity against CLZ, its active metabolite dmCLZ, and OLZ will alter kinetics parameters of enzyme maximum reaction velocity, enzyme affinity for the substrate, and reaction efficiency compared to wild-type UGT enzymes.

Specific Aim 3

The purpose of the study "Analysis of severe weight gain-inducing second-generation antipsychotic glucuronidation by hepatic UDP-glucuronosyltransferase variants,” was to explore the in vivo relationships between UGT genetic variants and CLZ, dmCLZ, and OLZ glucuronidation using a series of 115 HLM.

Hypothesis 3A: Functional variants and known promoter polymorphisms of UGTs whose wild-type enzymes have enzymatic activity against CLZ; its active metabolite dmCLZ, and OLZ will affect the glucuronidation activity of HLM.

Hypothesis 3B: HLM with variant UGTs will have different enzymatic activities compared to HLM with wild-type UGTs, as displayed by kinetic parameters such as reaction maximum velocity, enzyme affinity, and reaction efficiency.
Specific Aim 4

The purpose of the ongoing study "Phenotype:genotype study of second-generation antipsychotic metabolite levels, patient response, and UGT genotype," is to conduct a phenotype:genotype clinical research study examining the relationships between UGT genotypes and (i) SGA plasma concentrations, (ii) SGA metabolite plasma and urinary concentrations, and (iii) SGA efficacy and adverse effects.

Hypothesis 4A: Polymorphisms in UGT enzymes will correlate with SGA and SGA metabolite plasma and urine concentrations.

Hypothesis 4B: Higher SGA plasma concentrations and correspondingly lower SGA metabolite concentrations will correlate with risk of SGA adverse effects, including weight gain, diabetes, insulin resistance, and dyslipidemia.
Chapter 2

REVIEW OF LITERATURE

This chapter will address the literature relevant to the topics comprising this dissertation by discussing 1) The rise in use and mechanism of action of SGAs, 2) major adverse effects associated with SGAs, 3) reports of pharmacogenetic influences on SGA efficacy and adverse effect risk, 4) UGT enzyme gene organization, transcription, translation, cellular location, and function, and 5) the specific findings providing evidence of UGT modulation of SGA-induced adverse effect risk that underlie the following studies.

Overview of Second Generation Antipsychotics

History and Utilization

Antipsychotics are a group of psychiatric drugs that are effective and indicated for mental disorders presenting with prominent psychotic symptoms, including schizophrenia, bipolar disorder, and delusional disorder (3, 4). The first antipsychotic was introduced in the 1950s with the approval of chlorpromazine, which allowed for the systematic deinstitutionalization of people with severe mental illness from psychiatric hospitals and their reintegration into the community. The success of chlorpromazine led to the development of other antipsychotic compounds and the discovery of antidepressants. These first antipsychotics, commonly called ‘neuroleptics’, all demonstrated clinical efficacy against the positive symptoms of schizophrenia, defined as symptoms that experienced by schizophrenic patients that non-schizophrenic individuals do not including delusions, disordered communication, and hallucinations, and were associated with
neurologic movement disorders, including EPS such as parkinsonism, dystonia, and tardive dyskinesia (29). In response, the Swiss pharmaceutical company Wander AG (49) developed a group of tricyclic compounds based on the chemical structure of the antidepressant imipramine described as ‘tricyclic antidepressants with neuroleptic properties (50). CLZ (Clozaril) was notable within this group because it was an efficacious antipsychotic with no associated movement disorders (51, 52).

CLZ was termed “atypical” as it lacked neurological side effects, which was a significant barrier to its widespread acceptance as the psychopharmacological dogma of the time dictated that extrapyramidal effects were closely associated with antipsychotic efficacy (51, 53). Further setbacks emerged when long term trials revealed that 1-2% of patients taking CLZ developed agranulocytosis, an acute condition involving a severe and dangerous leukopenia (lowered white blood cell count) (54), but it eventually gained FDA approval many years later after longitudinal studies revealed superior efficacy for patients resistant to earlier treatments and with the development of an aggressive monitoring plan to prevent agranulocytosis. The introduction of CLZ prompted other pharmaceutical companies to develop “atypical” antipsychotic medications. In order of FDA approval, these were, with their accompanying trade names in parentheses: risperidone (Risperdal), OLZ (Zyprexa), quetiapine (Seroqual), ziprasidone (Geodon), aripiprazole (Abilify), paliperidone (Invega), iloperidone (Fanapt), and asenapine (Saphris) (55).

Subsequently, the name ‘second-generation antipsychotic’ (SGA) was coined to describe CLZ and the antipsychotics deemed “atypical” due to their lack of extrapyramidal symptoms, and “first-generation antipsychotics” (FGA) was used to describe “typical” pre-CLZ antipsychotics that were associated with movement disorders.

Antipsychotic drugs are individually reviewed and approved by the FDA for specific conditions. Table 2-1 shows FDA-approved SGAs by condition (55).
Table 2-1 FDA approved SGAs by condition

<table>
<thead>
<tr>
<th>SGA</th>
<th>FDA approved conditions</th>
</tr>
</thead>
</table>
| Aripiprazole | 11/15/02 treatment of schizophrenia  
            | 9/29/04 treatment of Bipolar Disorder-associated acute manic or mixed episodes  
            | 3/1/05 maintenance therapy in Bipolar I Disorder  
            | 11/16/07 adjunctive treatment to treat patients with major depressive disorder  
            | 5/6/08 monotherapy in the acute treatment of bipolar disorder, manic or mixed, at a starting dose of 15 mg/day; adjunctive therapy added to lithium or valproate in the short-term treatment of bipolar disorder, manic or mixed, at a starting dose of 15 mg/day. |
| Asenapine | 8/13/09 acute treatment of schizophrenia in adults and the acute treatment of manic or mixed episodes associated with Bipolar I Disorder in adults  
            | 9/3/10 maintenance treatment of schizophrenia in adults and adjunctive therapy with either lithium or valproate for the acute treatment of manic or mixed episodes associated with bipolar I disorder |
| Clozapine | 9/26/89 treatment of treatment-resistant schizophrenia  
            | 12/18/02 treatment of schizophrenia or schizoaffective disorder at risk for emergent suicidal behavior |
| Iloperidone | 5/6/09 acute treatment of schizophrenia in adults |
| Lurasidone | 10/28/10 treatment of schizophrenia |
| Olanzapine | 9/30/96 treatment of psychotic disorders  
            | 3/17/00 treatment of manic or mixed episodes in bipolar disorder  
            | 11/9/00 maintenance of treatment response  
            | 7/10/03 in combination with lithium or valproate for the treatment of acute manic episodes associated with bipolar disorder  
            | 1/14/04 long-term treatment of bipolar I disorder |
| Paliperidone | 12/19/06 maintenance treatment of schizophrenia  
            | 7/31/09 extended-release injectable suspension for the acute and maintenance treatment of schizophrenia in adults |
| Quetiapine | 9/26/97 treatment of the manifestations of psychotic disorders  
            | 1/12/04 monotherapy in the treatment of acute manic episodes associated with Bipolar I disorder; adjunctive therapy with mood stabilizers (lithium or divalproex) in the treatment of acute manic episodes associated with Bipolar I disorder  
            | 10/20/06 treatment of major depressive episodes associated with bipolar disorder.  
            | 5/13/08 maintenance treatment for bipolar I disorder, as adjunctive therapy to lithium or divalproex. |
| Risperidone | 12/29/93 treatment of psychosis  
            | 12/4/03 monotherapy for short term treatment of acute manic or mixed episodes associated with Bipolar I Disorder; adjunctive therapy for short term treatment of acute manic or mixed episodes associated with Bipolar I Disorder  
            | 10/6/06 treatment of irritability associated with autistic disorder |
| Ziprasidone | 2/5/01 treatment of schizophrenia  
            | 8/19/04 monotherapy in the treatment of acute manic or mixed episodes in Bipolar I Disorder, with or without psychotic features  
            | 11/20/09 maintenance treatment of bipolar disorder, as an adjunct to lithium or valproate |
All SGAs are approved for the treatment of schizophrenia, except for CLZ, which is approved for treatment of refractory schizophrenia or suicidal symptoms of schizophrenia. Due to their initial approval for treatment of psychosis, risperidone, quetiapine and OLZ are approved for secondary psychosis associated with Alzheimer's disease and dementia. All SGAs are approved for use in bipolar mania except CLZ, iloperidone, and paliperidone. SGAs are used off-label for treatment of chemotherapy-induced nausea and vomiting, anorexia, depression, obsessive-compulsive disorder, posttraumatic stress disorder (PTSD), personality disorders, Tourette's syndrome, autism, and agitation in dementia (56).

According to the most recently published analysis of Medical Expenditure Panel Survey (MEPS) Data, an estimated 3.21 million patients received antipsychotic agents in 2003, of which 71% were SGAs (2.29 million patients) (57). The most frequently used SGAs were risperidone, OLZ, and quetiapine. An estimated $2.82 billion was spent on antipsychotic agents with SGAs accounting for 93% of these expenditures, or $2.63 billion. The average cost of SGAs is about four-times that of FGAs, costing $163.70±11.91 compared to $39.89±4.72, respectively, with quetiapine being the least expensive ($101.84±10.00) and aripiprazole the most expensive ($312.36±64.59). OLZ ($232.80±28.06) and risperidone ($140.70±10.77) constituted 65% of the antipsychotic expenditures. Medicaid (46%) and out-of-pocket payments (32%) accounted for most of the SGA expenditures, while private insurance (19%) and other sources (3%) accounted for the remaining expenditures (57).

Mechanism of Action of Second Generation Antipsychotics

Table 2-2 shows approximate neurotransmitter receptor binding affinities (expressed as $K_i$) for the FDA-approved SGAs and select FGAs.
Table 2-2 Approximate receptor binding affinities for SGAs and select FGAs expressed as an equilibrium constant (Ki, nM)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Apomorphone</th>
<th>Ziprasidone</th>
<th>Haloperidol</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₁</td>
<td>8.9 73</td>
<td>216 262</td>
<td>31 620</td>
</tr>
<tr>
<td>D₂</td>
<td>44 90 &gt;1000</td>
<td>9 30 &gt;1000</td>
<td>14 56</td>
</tr>
<tr>
<td>5-HT₁A</td>
<td>1.7 8.6</td>
<td>- 6.4</td>
<td>- 420</td>
</tr>
<tr>
<td>5-HT₁B</td>
<td>10.2 2.6</td>
<td>1.1 0.36</td>
<td>1.5 0.52</td>
</tr>
<tr>
<td>5-HT₁C</td>
<td>9.8 10.5</td>
<td>12 415</td>
<td>11 63</td>
</tr>
<tr>
<td>5-HT₂</td>
<td>39 9.9 69</td>
<td>63.1 0.5 57</td>
<td>- 10</td>
</tr>
<tr>
<td>α₁A</td>
<td>8.9 7</td>
<td>48 19</td>
<td>23 13</td>
</tr>
<tr>
<td>α₂A</td>
<td>8.9 8</td>
<td>2.8 41</td>
<td>230 23</td>
</tr>
<tr>
<td>α₂B</td>
<td>4.8</td>
<td>8.5</td>
<td>-</td>
</tr>
<tr>
<td>α₂C</td>
<td>-</td>
<td>9.1</td>
<td>-</td>
</tr>
<tr>
<td>H₁</td>
<td>61 9.0 6</td>
<td>437 &gt;1600</td>
<td>7 27</td>
</tr>
<tr>
<td>H₂</td>
<td>8.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M₁</td>
<td>&gt;1000 5.6 19</td>
<td>&gt;1,000 &gt;1,000 1.9 &gt;1,000</td>
<td>120 2800 300</td>
</tr>
<tr>
<td>M₂</td>
<td>- 4.5</td>
<td>- 2150</td>
<td>-</td>
</tr>
</tbody>
</table>

Pharmacokinetics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₁/₂ (h)</td>
<td>75</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>87</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>3-5</td>
</tr>
</tbody>
</table>

Both FGAs and SGAs antagonize dopamine (DA) receptors, and blockade of D₂ receptors in mesolimbic nuclei, especially the nucleus accumbens, stria terminalis, and the extended amygdala, initiates the diminishing of psychotic symptoms (58-61). The depolarization inactivation hypothesis postulates that the long-term efficacy of antipsychotics is mediated by D₂ receptor blockade in the striatum, nucleus accumbens, and prefrontal cortex, which initially results in compensatory increases in dopaminergic activity in the substantia nigra compacta and ventral tegmentum, followed by a gradual decrease in dopaminergic activity leading to complete...
inactivation of DA neuron firing in these regions (61-64). Over-antagonism of DA receptors results in antipsychotic-induced EPS, as evidenced by D₂ receptor occupancies greater than 70% being associated with EPS and FGAs as a group having a higher D₂ dissociation constant (Kₐ, ligand concentration at which half of receptors are occupied).

In comparison to FGAs, SGAs exhibit greater affinity for serotonergic (5-HT) receptors than dopaminergic receptors. CLZ, risperidone, OLZ and ziprasidone occupy more than 80% of cortical 5-HT₂₅ receptors in the therapeutic dose range in humans, while occupying ≤70% of D₂ receptors (65). Preferential binding to 5-HT₂₅ receptors is coined the dopamine-serotonin antagonism theory, which is the prevailing dogma to explain SGAs superior efficacy and reduced EPS (65-68) and this measure is commonly used to delineate SGAs from FGAs (65, 67-72). 5-HT₂₅ receptor antagonist monotherapy fails to ameliorate psychotic symptoms, suggesting that 5-HT₂₅ receptor antagonism may be more important for preventing EPS than contributing to the efficacy of SGAs (73). Still, the beneficial effects of 5-HT₂₅ receptor antagonism on EPS can be overcome with high levels D₂ receptor occupancy, as evidenced by high dose risperidone-induced EPS (74). These results may be explained by the “fast-off D₂ theory” which hypothesizes that antipsychotics with a higher D₂ receptor Kₐ allow for physiological DA transmission, thereby permitting an antipsychotic effect without EPS and hyperprolactinemia (75, 76). In order of Kₐ, quetiapine > CLZ > OLZ > ziprasidone > risperidone (76-78). While the fast-off D₂ theory may explain why SGAs like risperidone can elicit EPS at higher doses, it cannot account for the greater therapeutic efficacy of CLZ compared to other SGAs, particularly in the management of treatment-resistant schizophrenia, all antipsychotics have not been tested to see if they support this theory, and it remains unclear how long an antipsychotic drug must bind to the D₂ receptor to maximize therapeutic efficacy while minimizing the risk of D₂-related side effects (79).
The SGAs have multiple sites of action other than D₂ and 5-HT₂A receptors, including dopamine (D₁, D₃, D₄), serotonin (5-HT₁A, 5-HT₂C, 5-HT₃a, 5-HT₆, 5-HT₇), norepinephrine (α₁, α₂) muscarinic cholinergic and histamine receptors. DA levels in the prefrontal cortex and the striatum are inversely correlated, and low prefrontal cortex and high striatal DA levels are commonly seen in schizophrenic patients (80, 81). SGA partial agonist activity at 5-HT₁A receptors may contribute to SGA efficacy in treating anxiety, depression, and in cognitive and negative symptoms of schizophrenia by enhancing prefrontal cortex DA release (82). CLZ, OLZ, and ziprasidone, but not haloperidol or risperidone, preferentially augment DA and norepinephrine (NE) release in the prefrontal cortex relative to subcortical areas, which may be related to their potential efficacy for negative symptoms and cognitive dysfunction of schizophrenia (83). Furthermore, activating inhibitory 5-HT₁A autoreceptors block striatal D₂ receptors (84), which potentiates the dopaminergic antagonistic effects of antipsychotics (85) and counteracts the development of EPS.

The prefrontal cortex contains high densities of 5-HT₁A and 5-HT₂A receptors located on affrents to and on pyramidal neurons, which are the primary excitation units implicated in cognitive ability in the prefrontal cortex and normal motor control in the corticospinal tract (86). It has been suggested that 5-HT₂A receptor activation increases the release of the excitatory neurotransmitter glutamate onto pyramidal cells (87), whereas serotonin, possibly via activation of 5-HT₁A receptors, inhibits the release of glutamate (88). Thus, compounds with 5-HT₂A antagonism and/or 5-HT₁A agonism, such as CLZ, could regulate the physiological balance between excitatory and inhibitory inputs onto prefrontal pyramidal neurons (86, 89). Some SGAs, particularly ziprasidone, increase serotonin activity in the frontal cortex through their affinity for the serotonin transporter (90, 91). Some of the SGAs, but not FGAs, increase acetylcholine (Ach) release in the prefrontal cortex, which could be a possible factor contributing to improve cognition in schizophrenia (92).
Noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists such as phencyclidine (PCP) and ketamine induce positive, negative and cognitive schizophrenia-like symptoms, leading to the hypothesis that NMDA receptor hypofunction contributes to the pathophysiology of schizophrenia (93-97). SGAs attenuate the effects of NMDA antagonist-induced hypofunction in both cellular and behavioral experiments, suggesting that SGA therapeutic efficacy is mediated in part by counteracting NMDA receptor hypofunction (98-103). For example, CLZ and OLZ, but not the FGAs haloperidol or raclopride, inhibit the electrophysiological effects of PCP in brain slices (101, 104, 105) and attenuate NMDA antagonist-induced deficits in prepulse inhibition (defined as the neurological phenomenon in which a weaker prestimulus (prepulse) inhibits the reaction of an organism to a subsequent strong startling stimulus (pulse), which has been found to be disrupted in schizophrenia) (98, 106) and social behavior (99). In addition, ketamine-induced brain metabolic activation is blocked by acute administration of CLZ and OLZ, but not by haloperidol, in rats (100, 102). Chronic administration of haloperidol blocks PCP-induced deficits in prepulse inhibition (107, 108) and ketamine-induced brain metabolic activation (109). Thus, adaptive changes elicited by chronic treatment with both the FGAs and the SGAs appear to attenuate the effects of NMDA antagonists (109). The mechanism by which SGAs attenuate NMDA antagonists is not understood as none of the SGAs have direct affinity for the NMDA receptor or any of the glutamate receptors. For CLZ, hippocampal NMDA receptor currents can be potentiated through M₁ receptor activation via the biologically active metabolite of CLZ, dmCLZ, which is a potent, allosteric agonist at muscarinic M₁ receptors (110). Thus, the unique therapeutic profile of CLZ may be, in part, attributed to dmCLZ through potentiation of NMDA receptor function mediated by M₁ receptors.
There have been conflicting reports of changes in glutamate receptor binding sites in various brain regions after chronic administration of antipsychotics in animals (111-117) and inconsistent findings in the gene expression of different glutamate receptor subunits following long-term treatment with antipsychotics (118-125). These discrepancies appear to be due to different treatment regimens, brain regions examined and the method of assessment. Thus, it is unclear whether such changes reflect increased or decreased function of different glutamate receptors after chronic antipsychotic treatments and further studies are needed to determine whether SGAs inhibit the effects of NMDA antagonists by molecular modification of glutamate receptors or alter other neurotransmitter–glutamate interactions.

DA, GABA, and glutamate are three key neurotransmitter systems implicated in the pathophysiology of schizophrenia, alterations in other neurotransmitter systems have been suggested and include serotonin (126), acetylcholine (127), muscarinic receptors (128, 129) and nicotinic receptors (130, 131), and norepinephrine (132, 133), all of which SGAs have been shown to bind to or modulate.

Prolonged administration of SGAs changes signaling mechanisms and gene expression, which may contribute to their antipsychotic effects. SGAs affect the expression and activity of signaling messengers downstream of D₂ receptors such as protein kinase A (PKA), cyclic-adenosine monophosphate (cAMP), and DA- and cAMP-regulated phosphoprotein (DARPP)-32 (134-137). SGAs affect the mitogen-activated kinase pathway (MAKP), which is involved in glutamate signaling, growth and development, and memory, the PKB/glycogen synthase kinase 3 pathway, which is involved in regulating many cellular processes including apoptosis and cell proliferation, and the β-arrestin-2-dependent pathway (138). SGAs affect transcription of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor.
(NGF). Finally, SGAs have been reported to affect transcription of the glutaminergic system (138), neuroactive steroids (139), the GABA system (139), and other targets involved in neurogenesis, neuronal plasticity, mitochondrial biogenesis, cell energetics, and antioxidant defense enzymes (138-140), although the implications of these changes in SGA efficacy is unclear.

**Efficacy and Adverse Effects**

**Efficacy**

There is growing debate in the field of psychiatry as to whether SGAs as a group are more efficacious compared to FGAs (141, 142). In general, antipsychotic treatment is rather unsuccessful; in the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) in which schizophrenic patients were randomized to therapy with a FGA or SGA and followed for up to 18 months, over 74% eventually discontinued their medication due to lack of efficacy or tolerability(140), with OLZ having the longest time until treatment discontinuation and better treatment adherence (143). Clinical trials comparing efficacy of FGAs to SGAs fail to show consistent benefits in measures such as the Positive and Negative Syndrome Scale (PANSS), BPRS (Brief Psychiatric Rating Scale), neurocognition, or quality-adjusted life years (QALYs) (139). However, there is evidence that certain SGAs can improve specific measures of diseases, such as having higher efficacy against the negative symptoms (poverty of thought, blunted affect, withdrawal) of schizophrenia than FGAs, and lower incidences of the classic FGA-associated adverse effects of movement disorders and prolactin elevation, overall leading to lower discontinuation rates (143, 144). Of note, CLZ is the only SGA to be proven more efficacious on
patients with treatment-resistant schizophrenia (139, 145). However, it is associated with the major, life-threatening side effect of agranulocytosis in 1% of patients and thus carries a clinical burden of frequent hematologic monitoring.

Given the lack of efficacy in so many patients, it has been suggested that SGA treatment should be personalized to an individual, determining which drugs would be efficacious before starting treatment or following drug measures of efficacy during the beginning of treatment to diminish side effects and improve therapeutic outcomes. Efficacy has been associated with receptor binding in the brain and also plasma concentrations. It is recognized that plasma concentrations of the SGAs are an accurate measure of brain concentrations, which can allow one to predict the receptor occupancy in the brain (19, 146). Therapeutic drug monitoring (TDM) has been suggested for a number of SGAs, which is a cheaper measure of SGA activity that positron emission tomography (PET) scanning of receptor occupancy, and optimal plasma concentrations have been noted for a number of SGAs (19, 21). Regardless, some patients cannot achieve therapeutic plasma levels of SGAs or overshoot these levels even at low doses of SGAs, suggesting that drug metabolism is an important determinant of drug efficacy in patients.

**Adverse Effects of Second Generation Antipsychotics**

Table 2-3 outlines the adverse effects associated with antipsychotic treatment and which SGAs are associated at a higher incidence with which adverse effects.
Table 2-3 Commonly reported adverse effects of second-generation antipsychotics

<table>
<thead>
<tr>
<th>Drug</th>
<th>Daily dose (mg)</th>
<th>EPS</th>
<th>TD</th>
<th>Hyperprolactinemia</th>
<th>Weight gain</th>
<th>Glucose intolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-generation antipsychotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>50–450</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>3–6</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>6–48</td>
<td>++++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Second-generation antipsychotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td>150–600</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Risperidone</td>
<td>2–6</td>
<td>±~±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>10–20</td>
<td>±~+</td>
<td>+</td>
<td>±</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>150–750</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>80–200</td>
<td>±~±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Amisulpride</td>
<td>50–800</td>
<td>±~+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>6–30</td>
<td>±~±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Perosprone</td>
<td>12–48</td>
<td>±~±</td>
<td>NA</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Blonanserin</td>
<td>8–24</td>
<td>±~±</td>
<td>NA</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

EPS: Extrapyramidal signs; NA: Information not available; TD: Tardive dyskinesia. Data from [65, 147, 148].

Movement Disorders

Classically, antipsychotic treatment is associated with the development of movement disorders, including EPS, parkinsonism, and TD. It is thought that antipsychotics induce movement disorders through their high affinity and antagonism of D₂ receptors in the striatum (65, 147, 148). The incidence of movement disorders is lower with SGAs than FGAs, likely due to their increased Kᵅ at D₂ receptors, preference for prefrontal cortex over striatal activation, and higher affinity for serotinergic receptors than dopaminergic receptors (65, 149). However, some SGAs are associated with this side effect, most notably risperidone, which has the lowest Kᵅ for D₂ receptors out of the family of SGAs (76, 77).
Prolactin Elevation

Another classically-associated adverse effect of antipsychotics, prolactin elevation is thought to be mediated by DA receptor antagonism in the tuberoinfundibular pathway (150). SGAs are associated with a lower incidence of prolactin elevation than FGAs, except for aripiprazole and risperidone (151, 152).

Weight Gain

Clinically significant (>7% baseline body weight) weight gain is a well-reported adverse effect of SGA treatment and an important cause of therapy non-adherence (8, 153, 154). A higher proportion of patients with schizophrenia meet the clinical criteria for obesity compared to the general population (42% versus 27%) (155, 156) and it is thought that SGA therapy contributes to this number. Weight gain is associated with increased risk of diabetes, cardiovascular disease, and subsequently a higher risk of mortality (6, 157). Blockade of histamine H1 (158) or serotonin 5-HT2C receptors has been associated with weight gain observed with some SGAs (159). Weight gain can occur during use of any antipsychotic, but is most prominent with the SGAs CLZ and OLZ, followed by risperidone, quetiapine, amisulpride and zotepine which show low to moderate levels of mean weight gain (156, 160). Ziprasidone and aripiprazole treatment are generally associated with minimal mean weight gain (Table 2-3).

Among the atypicals, CLZ appears to have the greatest weight gain liability (160, 161). Some patients may gain as much as 50 kg over a 1-year treatment period and across the literature 13–85% of patients treated with CLZ had an associated increase in weight (162). The cumulative incidence of all patients reaching ≥20% overweight, a significant long-term health risk, was >50% (163, 164). Significant weight gain can undermine compliance, leading to relapse, and can also cause significant psychological and medical morbidity. Considerable weight gain increases risk of obesity-related diseases and comorbidities such as type II diabetes mellitus, hypertension,
CVD, respiratory dysfunction and some types of cancer. All of these are associated with significant mortality (5, 6, 165-168).

There appears to be considerable variability among individuals with respect to the ability of an antipsychotic to induce weight gain, i.e. not all patients treated with CLZ gain weight. Thus, the side-effect of weight gain occurs in only a proportion of treated patients who are predisposed to this side-effect. It is likely that this variability in weight-gain risk is determined by a combination of genetic and environmental factors (169-172). The genetic factors may include pharmacokinetic (i.e. factors involved in the metabolism and elimination of the drug from the body) as well as pharmacodynamic (i.e. factors at the direct site of action of the drug within the body) elements. Genetic variation in pharmacodynamic factors such as brain receptors involved in satiety control may result in some patients having receptors with higher affinity for the medication and may allow prediction of those patients who are most likely to respond to the drug or develop side effects.

Genetic differences in pharmacokinetic factors such as drug-metabolizing enzymes may reduce or increase their activity, resulting in altered plasma levels of the medication, and this may also allow prediction of good response and/or propensity to side-effects. Numerous studies have associated weight-gain with SGA plasma concentrations and plasma concentrations are a good measure of brain SGA concentrations (19, 43). Optimal plasma levels that are less associated with weight gain have been reported for SGAs, however, plasma levels cannot be predicted by administered dose, indicating that DMEs are determinants of an individual’s plasma level and drug response.

Metabolic Syndrome and Diabetes

SGAs are associated with development of metabolic syndrome and diabetes (5, 173), with OLZ and CLZ having the highest association with risk (174). The prevalence of diabetes in
2 times that of the general population (175) and, in the CATIE study, metabolic syndrome prevalence was approximately twice that of age-matched general population controls (140). A meta-analysis of trials comparing SGAs to FGAs where diabetes was an outcome, a slight increase in risk of development of diabetes was observed for SGAs (RR=1.32) (176). It is generally accepted that the incidence of metabolic syndrome and diabetes in patients taking SGAs is a result of SGA-induced weight gain, as increased adiposity is associated with decreased insulin sensitivity and changes in plasma glucose and lipid levels. This idea is supported by the trend that weight gain liability of a specific SGA parallels differing relative risk of insulin resistance, dyslipidemia and hyperglycemia (5). Interestingly, OLZ produced a significantly greater increase in glucose levels from baseline to endpoint than amisulpride, aripiprazole, quetiapine, risperidone, and ziprasidone. There were no statistically significant differences in glucose changes between aripiprazole and risperidone, CLZ and OLZ, CLZ and risperidone, quetiapine and risperidone, quetiapine and ziprasidone, risperidone and sertindole, and risperidone and ziprasidone (160). There is a paucity of literature examining the importance of SGA receptor occupancy or plasma concentrations in relationship to metabolic syndrome or diabetes and this is an important hole in the literature.

Other theories exist regarding the direct effects of SGAs on glucose metabolism and their potential impact on metabolic syndrome or diabetes. For example, some antipsychotics inhibit glucose transport in erythrocytes (177) and cultured neuronal and muscle cell lines (178-180). Among the SGAs, risperidone, ziprasidone, and CLZ effectively block glucose transport, whereas OLZ and quetiapine are somewhat less effective (181-183); aripiprazole has not been evaluated to date. The drugs seem to bind directly to the glucose transporter and affect GLUT1 and GLUT3 transport and, with longer incubation periods, increase the expression of GLUT1 and GLUT3 in cells, perhaps as a consequence of relative glucose deprivation (180, 184). Finally, OLZ has been shown to enhance cellular uptake of glucose (179, 182). The risk of diabetes with SGAs has led
to a consensus statement from the American Diabetes Association (ADA) and the American Psychiatric Association (APA) regarding monitoring patients chronically administered these agents (185). These recommendations are summarized in Table 2-4.

Table 2-4 Monitoring of weight-gain and CVD risk factors for patients taking SGAs

<table>
<thead>
<tr>
<th>Monitor:</th>
<th>baseline</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>Every 3 months</th>
<th>Every 12 months</th>
<th>Every 60 months</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
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<tr>
<td>Family history</td>
<td>X</td>
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</tr>
<tr>
<td>Weight (body mass index)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
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<td></td>
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<tr>
<td>Waist circumference</td>
<td></td>
<td>X</td>
<td></td>
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<tr>
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<td></td>
<td>X</td>
</tr>
</tbody>
</table>

ADA and APA recommendations for monitoring a patient chronically taking a SGA. More frequent monitoring may be required based on the family history and/or individual risk profile of the patient. An asymptomatic patient exhibiting an abnormal fasting glucose or lipid level should receive further testing.

**Lipid Elevation**

Individuals with severe mental illness have approximately 1.5 to 2 times the general population prevalence of diabetes, dyslipidemia, hypertension, and obesity (5, 140, 185). SGAs induce dyslipidemia in patients chronically taking these drugs, with increases most notable in triglyceride levels (186). Low potency FGAs and the SGAs CLZ, OLZ, and quetiapine are associated with a higher risk of dyslipidemia (160, 187-189).

**Heart Disease**

Approximately 5-10% of the US population has a severe mental illnesses such as schizophrenia, bipolar disorder, and depression (190) and it is estimated that these patients lose 25 or more years of life expectancy primarily due to CVD, not suicide (191). Cardiovascular
(CV) illness-related deaths are at least 6-fold higher in schizophrenia (192, 193) and are the largest contributor to the excess mortality observed in the disorder (194, 195). Obesity, diabetes and metabolic syndrome, and lipid elevation are all risk factors for heart disease. The high prevalence of modifiable CVD risk factors can be explained in part by under-diagnosis and under-treatment and in part by contributions related to the mental illness, including effects of the medications used for treatment, some of which have unfavorable effects on various metabolic risk factors for CVD (6). It is unclear if SGAs worsen this risk in addition to the metabolic side effects.

**Plasma Concentrations, Efficacy, and Adverse Effects**

Plasma SGA levels are correlated with clinical effects, although there is considerable variability in the response achieved at any given drug concentration (17, 20, 21). Higher plasma concentrations were correlated with significant clinical response as measured by the BPRS. However, patients with higher plasma concentrations also had a 2-fold increase in adverse effect risk (17, 42). BPRS scores related to improvement of suspiciousness, hallucinations, and blunted affect were significantly correlated with plasma OLZ concentration in a Japanese population of schizophrenic patients (22). Interestingly, during coadministration of OLZ and lamotrigine (LTG), which is primarily metabolized by UGT1A4, the area under the concentration vs. time curve, from 0-24 hours or in LTG plasma concentrations at baseline or at 24 hours was observed (196). However, coadministration with probenicid, another drug metabolized by UGT1A4, significantly altered plasma pharmacokinetic parameters compared with OLZ administered alone, showing the importance of UGT metabolism in OLZ clearance (197). Variation in intra-individual plasma CLZ levels has been shown to predict rehospitalization of schizophrenic patients (198), but this may be a measure of patient compliance.
Pharmacogenetics of Second-Generation Antipsychotics

Genetic variation plays an important role in interindividual differences in medication response and toxicity. There is considerable variability among individuals in SGA-induced weight gain; only a proportion of treated patients who are predisposed to this adverse effect gain weight. Likely, this variability is determined by a combination of genetic and environmental factors. The genetic factors may include pharmacodynamic (factors at the direct site of action of the drug within the body) as well as pharmacokinetic (factors involved in the metabolism and elimination of the drug from the body) elements.

Antagonism of SGA target receptors (e.g. muscarinic, histaminergic, 5-HT_{1a} and 5-HT_{2c}) has been associated with weight gain (156, 158, 199, 200) and it is thought that genetically determined differences in receptor binding, response, and expression further contribute to interindividual differences in SGA efficacy and weight gain.

Pharmacodynamic Genetic Variability

Pharmacodynamics is the study of a drug’s biochemical and physiological effects on the body and their mechanisms of action. Receptor binding (including receptor sensitivity), post-receptor effects, and chemical interactions with other endogenous or exogenous compounds are all pharmacodynamic mediators. A drug's pharmacodynamics can be affected by polymorphisms in genes involved in eliciting the drug’s physiological effect by altering receptor affinity, expression, or activity. Many studies have examined the importance of genetic variation in SGA-target receptors and their influence on drug efficacy and adverse effects. Studies have been categorized based on their receptor system.
Dopaminergic System

Dopaminergic mechanisms are involved in modulating reward pathways associated with food consumption. Dopamine receptor agonists (D\textsubscript{1}/D\textsubscript{2}) are involved in normalizing hyperphagia, body weight gain, hyperglycemia and hyperlipidemia in genetically obese (ob/ob) mice (201). Obese individuals have a decreased availability of dopamine (D\textsubscript{2}) receptors in proportion to their body mass index (202). The D\textsubscript{4}-receptor has also been associated with obesity in humans (203). An association between the 48 base pair variable number tandem repeat (VNTR) polymorphism in the D\textsubscript{4}-receptor and SGA-induced weight gain has been reported (204). Patients with less than seven repeats experienced less weight gain during antipsychotic treatment than patients with more than seven repeats (0.38 kg/m\textsuperscript{2} vs. 0.89 kg/m\textsuperscript{2} respectively, p 0.003); an association that was particularly strong in males. This result could not be confirmed for CLZ-induced weight gain (205). However, the study was not primarily designed to examine the association between D\textsubscript{4}-receptor polymorphisms and CLZ-induced weight gain and may have been underpowered. Patients on long term SGA treatment carrying the rs4436578-C/C genotype in the D\textsubscript{2} receptor had a higher risk of more than 7% weight gain than patients with the C/T or T/T genotype (odds ratio 3.36; 95%CI 1.62-7.00) (206). However, none of these associations have been confirmed.

Serotonergic System

A large body of evidence supports a role for the serotonin system in regulating feeding behavior along with other genes (207). 5-HT\textsubscript{2C} and 5-HT\textsubscript{1A} receptors are localized with high density in the satiety control center of the medial hypothalamus and it is thought that modulation of 5-HT receptor pathways in this center contribute to weight gain (208, 209). Pharmacological treatment with serotonin agonists results in decreased feeding while serotonin antagonists increase feeding (210-212), specifically 5-HT\textsubscript{1} agonists result in hyperphagia, while 5-HT\textsubscript{2} agonists result in hypophagia (213, 214). Knockout mice lacking 5-HT\textsubscript{2C} receptors (215) are
overweight compared with wild-type mice, and, based on paired feeding analysis, this appears to be due to increased feeding as opposed to metabolic changes. CLZ and OLZ are potent 5-HT$_{2C}$ antagonists and 5-HT$_{1A}$ agonists, which would suggest that modulation of the 5-HT pathways is, in part, how they illicit weight gain.

Several studies have focused on the 5HT$_{2C}$ -759C/T promoter polymorphism and SGA-induced weight gain. The majority report that the variant T-allele is associated with less SGA-induced weight gain (159, 209, 213, 214, 216-221), whereas some were unable to confirm this association (212, 215, 220, 222-224) or reported an association in the opposite direction (208, 225). Most studies that were inconclusive showed trends towards a protective effect of the T-allele. All studies investigating the -759 C/T polymorphism in a first-episode schizophrenic population reported a protective effect of the T-allele (159, 213, 214, 226). A meta-analysis confirmed the association between the T-allele and less SGA-induced weight gain in a fixed-effect model (227). Significance of this association was lost due to heterogeneity of the studies if the more appropriate random-effects model was chosen for analysis (odds ratio 2.29; 95%CI; 0.98-5.36). The association seems to be particularly strong in male patients, which is reasonable as the 5-HT$_{2c}$ gene is X-linked. The genetic association with SGA-induced weight gain is probably most profound in first treatment populations because there is no previous weight gain due to other antipsychotic treatment (219). Electromobility shift assay studies of the -759 C/T promoter polymorphism in the rat hypothalamus revealed altered DNA-protein interactions with the weight gain-resistant allele (759 T). This disruption in formation of two complexes suggested disruption of DNA-protein interactions as a mechanism by which 5-HT$_{2C}$ expression is perturbed leading to an influence on antipsychotic-induced weight gain (228). Other polymorphisms in the 5-HT$_{2c}$ gene, including the Cys23Ser, -697 G/C, -997 G/A and -1165 A/G polymorphisms, have been associated with SGA-induced weight gain (219, 221, 223), but these associations have not been confirmed.
5-HT\textsubscript{2A} -102 T/C and 5-HT\textsubscript{6} -267 C/T polymorphisms were investigated for associations with antipsychotic-induced weight gain. An association was reported between the -102T-allele in the 5-HT\textsubscript{2a} receptor with weight gain as was an association between the -267 T/C and C/C genotype of the 5-HT\textsubscript{6} receptor and weight gain (216, 223), however, more evidence is necessary to make definitive statements regarding their role in SGA-induced weight gain.

*Adrenergic System*

Adrenergic $\alpha_1$, $\alpha_2$ and $\beta_3$-receptors stimulate intracellular lipolysis and increase basal metabolic rate by increasing the expression of mitochondrial uncoupling proteins (UCP 1-3) (170). Stimulation of the UCP-pathway by $\alpha_1$, $\alpha_2$ and $\beta_3$-receptor agonists results in less weight gain.

The $\alpha_2a$-receptor -1291C/G polymorphism was associated with CLZ- or OLZ-induced weight gain (229-231). In an Asian population, the G-allele was associated with greater than 7% (229; 8.45 vs 2.79kg, $p=0.023$) or 10% (230; odds ratio 2.58 (95% CI 1.21-5.51)) weight gain. However, in a Caucasian population, the C-allele was associated with greater weight gain than the G/G-genotype (3.73 kg vs. 0.23 kg, $p=0.013$) (231). Thus, the role of this polymorphism in SGA-induced weight gain is unclear.

The $\beta_3$ receptor is involved in the phenotype of obesity and $\beta_3$ agonists are being developed to treat patients with obesity (232, 233). The Trp64Arg polymorphism in the $\beta_3$ receptor has been associated with SGA-induced weight gain (223). Carriers of the Arg/Arg genotype had a significant ($p=0.024$) association with OLZ-induced weight gain. A trend for this association has also been reported (170). However, the Arg-allele is a low prevalence allele.
Histaminergic System

Histamine (H₁) receptor antagonism causes increased feeding and weight gain (159, 220, 221). H₁ receptors are densely concentrated in the hypothalamic regions important to regulating satiety: The ventromedial nucleus and the paraventricular nucleus (234). An exponential relationship between the maximum amount of weight gained while being treated with an antipsychotic and affinity for the H₁ receptor has been reported (161), with CLZ and OLZ having the greatest affinities. The histaminergic system has been linked to SGA-induced weight gain (199). Three studies investigated the association between polymorphisms in the histamine receptor and antipsychotic-induced weight gain. None of the studies found a significant association between polymorphisms in the H₁, H₂ and H₃-receptor and SGA-induced weight gain (170, 223, 234), although these studies had limited power due to small genotype groups.

Leptin

Leptin is a 16-kiloDalton (kDa) protein hormone that has an important role in body weight regulation. Leptin is stored in adipocytes and serum leptin concentration is positively correlated with the amount of adipose tissue. After being secreted by adipocytes, leptin affects the hypothalamus, resulting in a reduced food intake and fat storage and promoting energy expenditure (235). In several studies, the G-allele of the -2548A/G polymorphism was linked to an increased susceptibility to SGA-induced weight gain (214, 235-237). One study could not confirm this association (221) while another reported a protective effect of the GG-genotype for weight gain and obesity in children and adolescents (238). The rs4731426 C/G polymorphism was found to be moderately associated with median weight gain and significantly associated with extreme weight gain (239). A strong interaction between the leptin -2548 A/G and 5-HT₂C -759 C/T genotype and their effects on antipsychotic-induced weight gain has been reported in multiple studies (214, 240-242).
Pharmacokinetic Genetic Variability

Genetic differences in pharmacokinetic mediators such as DMEs and drug transporters affect drug inactivation and clearance, are an index of plasma and urine drug levels, and may provide a predictive measure of good response or propensity to adverse effects. SGAs are extensively metabolized (243). SGA brain concentrations are predicted well by their plasma concentrations rather than by dose (224) and plasma concentrations of antipsychotic drugs correlate well with receptor occupancy (19, 159, 208, 209, 215, 219-221). DA and 5-HT receptor occupancy in the brain as determined by PET imaging is predictive of SGA efficacy or adverse effects such as weight gain or tardive dyskinesia (68, 244, 245). Differences in SGA-induced weight gain have been correlated to higher plasma concentrations of the drug (246). Interindividual variability and propensity for weight gain are independent of dose but correlate very well with plasma concentration (31), reflecting differences in DME expression or activity. Studies examining DME and drug transporter genetic predictors of pharmacokinetic variability are reviewed here and have been categorized based on their enzyme family.

Cytochrome p450 Enzymatic Complex

Cytochrome P450 (CYP450) enzymes are part of phase I metabolism and mediate oxidation, reduction, or hydrolysis reactions that introduce a functional group (-OH, -SH, -NH2, or -COOH) into the drug molecule. A number of CYP450 have been studied for association with SGA plasma concentrations or outcomes, including CYPs 2D6, 1A2, 3A4. CYP2D6 is thought to be responsible for 25% of all drug metabolism (247) and is a major metabolic pathway of many antidepressants and a number of classical antipsychotics (247-249). More than 100 polymorphisms of the CYP2D6 gene are described (250). Of these, four polymorphisms (*3, *4, *5 and *6) are responsible for most inactive alleles (98%) in Caucasians resulting in the poor
metabolizer (PM) phenotype (251). Some gene duplications (or more rarely multiplications) are responsible for an ultra metabolizer (UM) phenotype, specifically the CYP2D6*1XN, *2XN and *35XN duplications with an active allele in the other chromosome are associated with a CYP2D6 UM profile (252). 7–10% of Caucasians and 1–2% of Asians are PMs (249).

In one study, the PM heterozygote CYP2D6 *1/*3 or *1/*4 genotypes were reported to predict 49% of OLZ-induced weight gain over the 47-week study period (253), even though CYP2D6 is a minor metabolic pathway of OLZ; forming a fraction of the inactive OLZ-N-oxide (8.6% of total urine metabolites) (254). The PM CYP2D6 *10 allele is correlated with 0.8-1.1 kg of risperidone-induced weight gain (216), the *4 PM is positively correlated with a tandard increase in risperidone/9-OH-risperidone (The active metabolite of risperidone whose formation is primarily mediated by CYP2D6) plasma levels, and showed a trend towards increasing the odds ratio of tardive dyskinesia (255-258), however, CYP2D6 did not predict measures of efficacy such as PANSS. No association was found between CYP2D6 PMs and CLZ response (259). However, adjustments of CLZ therapeutic doses according to CYP2D6 genotypes have been suggested in the literature and by biotechnology companies, mostly due to the prevalence of CYP2D6 polymorphisms (260).

Several polymorphisms have been reported for the CYP1A2 gene, with the *1C, *1K and *11 variants showing decreased activity (261, 262). Smoking increases the activity of CYP1A2 (263) particularly in individuals with the *1C and *1D variants (264). CYP1A2 mediates most of the catalysis of CLZ to dmCLZ and, with CYP2C19 and CYP3A4.15Y17 also contributing (265-267), and transforms dmCLZ to an inactive metabolite as well. CYP1A2 polymorphisms did not significantly influence individuals’ CLZ metabolic capacity (268), although delayed response to CLZ have been observed in individuals with the UM phenotype compared to normal metabolizers (269, 270). Furthermore, a combination of high inducibility CYP1A2 alleles and smoking may result in higher metabolic ratios and reduced CLZ plasma levels (271). In these cases, an
adjustment of therapeutic dose has been suggested (31, 269, 270). The CYP1A2 *1C and *1D alleles were reported to result in higher CLZ concentration to dose (C/D) ratios and elevated insulin and lipid ratios (272). The CYP1A2 *7 allele was associated with high plasma CLZ concentrations (273). A statistically significant reduction in levels of CLZ (47%) and CLZ-N-oxide (31%) and a significant increase (185%) in the ratio of dmCLZ to CLZ levels were correlated with CYP1A2 activity as measured by the caffeine clearance rate (274, 275). CYP1A2 efficiency, as measured by the ratio of dmCLZ/CLZ, contributed to prediction of CLZ plasma levels by ±8% for every 0.1 change in the ratio (30).

Numerous variants of the CYP3A4 enzyme, which is involved in the metabolism of antipsychotics, have been described. The CYP3A4*17 and *18A polymorphisms display functional variability with a decreased or increased activity, respectively (276). To date, no reports of the associations of these variants with antipsychotic variability have been published despite being involed in the metabolism of numerous SGAs, perhaps due to their relatively low prevalence in caucasian asian, and african populations (277). No significant response associations have been reported with the polymorphic CYP3A5, an enzyme reported to contribute to antipsychotic metabolism (278-280). Several variants related to decreased activity of CYP2C9 and CYP2C19 have been detected (281, 282), and one CYP2C19 variant (*17) with increased transcription in vitro has been described (281). Some studies have reported a lower dose requirement with CYP2C19 polymorphisms (260), but no connection between these variants and level of response to psychotropic drugs have been reported. Interestingly, CYP2D19 *2/*2 poor metabolizers had a 2.3-fold higher CLZ concentration than non-*2/*2 extensive metabolizer individuals (283). A number of studies report that CYP3A4 inhibition increases CLZ plasma levels and side effects (266, 283-285). The CATIE trial examined a number of DME genotypes versus drug efficacy, safety and dosing and found no significant effects (286). However, this may be due to the sample size, study design, the genotypes that were studied, or the chosen outcomes.
**UDP-Glucuronosyltransferase Enzymes**

UGT enzymes are phase II DMEs that catalyze the conjugation of a UDP-sugar moiety to an -O, -S, or -N group on a xenobiotic which, in general, facilitates inactivation and excretion. The UGT1A4 *3 allele, corresponding to the codon 48 (Leu>Val) functional variant, was reported to be a significant predictor of OLZ plasma levels, resulting in a 5-fold reduction compared to the wild-type allele (36). However, this has not been confirmed in other studies. In vitro, this UGT1A4 variant resulted in a 2-fold increase in enzyme efficiency (V_{max}/K_M) against CLZ in formation of all CLZ-glucuronides. Total serum thyroxine (T4) levels decreased in patients taking quetiapine, possibly related to competitive metabolism of thyroid hormones and quetiapine by UGTs (287). T4 has been reported to be metabolized by UGTs 1A1, 1A3, 1A7, 1A8, 1A9, and 1A10, although not all UGTs were screened (288).

**P-glycoprotein Efflux Transporter**

The P-glycoprotein (permeability glycoprotein, PGP) efflux transporter is a membrane bound ATP-binding cassette (ABC) transporter in the multiple-drug resistance (MDR) subfamily involved in drug resistance (289-292). It is an ATP-dependent efflux pump for xenobiotic compounds with broad substrate specificity. PGPs are responsible for decreased drug accumulation in multidrug-resistant cells and often mediate the development of resistance to anticancer drugs. This protein also functions as a transporter in the blood-brain barrier. Female patients carrying the SNP at nucleotide 3435 (G>T) or the wild-type SNP at nucleotide 2677 (T), resulting in a lower PGP function, gained more weight with risperidone, but not with OLZ, compared to patients with the 3435-CC or 2677-GG genotype respectively (222). Carriers of the ABC B1 3435TT genotype have higher CLZ plasma concentrations than noncarriers for a given CLZ dose (283, 293) and ABC B1 3435CC patients require significantly higher daily doses of CLZ compared with CT and TT patients to achieve the same clinical benefit (293). The
c.1236C>T (exon 12) and c.2677G>T (exon 21) ABC B1 polymorphisms have been examined; similar to the ABC B1 3435CC genotype, individuals who had the ABC B1 2677GG genotype had significantly lower dose-normalized CLZ levels than those who were heterozygous or TT carriers (293). However, these associations have not been confirmed.

**Uridine Diphosphate-Glycosyltransferase Enzymes**

**UDP-Glycosyltransferase Family of Genes**

UDP-Glycosyltransferase (UGT) Enzymes are encoded by the UDP-glycosyltransferase superfamily of genes. The gene superfamily contains four UGT families, UGT1, UGT2, UGT3 and UGT8 (294). Members of the UGT superfamily have been named based on divergent evolution, with each gene given the root symbol UGT, followed by an Arabic number representing the family, a letter to denote the subfamily, and an Arabic number for the individual gene within that family or subfamily (294) (Figure 2-1). To date, 19 functional human UGT genes have been identified (295); the UGT1 and the UGT2 families both utilize UDP-glucuronic acid as its glycosyl donor, are responsible for the largest percentage of drugs metabolized< and will be the focus of this dissertation.
Figure 2-1 Phylogram of human UGT genes
UGT1A Family

The human UDP-glucuronosyltransferase 1 gene is located on chromosome 2q37 and spans a region of approximately 200 kb. The region contains 13 individual promoters and first exon sets and a shared set of exons 2–5 (Figure 2-2) (296).

Figure 2-2 Human UGT1 locus organization

The UGT1 family. The human UGT1 loci extends over approximately 200 kb. Each human exon 1 is represented by a coloured rectangle, labeled A1, A2, A3, etc., and its position relative to exons 2–5 is indicated. Exons 2–5, which are joined to each first exon in the mature transcript, are shown in grey. Pseudogene names end in the label P. The exons are not drawn to scale. From MacKenzie et al 2005.

The promoter and donor RNA splice site flank the 5’- and 3’-ends of each first exon and direct the synthesis of RNA transcripts containing the first exon, which is then spliced to the shared exons 2–5. Thus, 13 potential transcripts can be generated from the UGT1 locus, containing unique 5’ ends and first exons and identical exons 2-5 and 3’ ends. Each exon 1 spliced to exons 2–5 is regarded as a unique gene and named as UGT1A1, UGT1A2P, UGT1A3, and so on. Four of the 13 human first exons, UGT1A2P, UGT1A11P, UGT1A12P and UGT1A13P, contain TATA box mutations and are designated as pseudogenes. The UGT1A genes were numbered according to their upstream position relative to exons 2–5, with 1A1 being closest and 1A13P the most distant, before the UG1 locus had been completely sequenced and defined (294, 296). Thus, the correct 3’ to 5’ arrangement of first exons extending from exon 1A7 is different from the order implied in the designated UGT1A names: UGT1A9, 1A13P, 1A10, 1A8, 1A11P and
1A12P (Figure 2-2). The original UGT1A gene names were not changed to reflect their genomic arrangement because the names had become universally accepted (297).

Based on sequence homology, the first exons for 1A1 and 1A6 are approximately 50% identical to each other and to the polypeptides encoded by the first exons for 1A2P, 1A3, 1A4 and 1A5 (exon cluster 1) and 1A7, 1A8, 1A9, 1A10, 1A11P, 1A12P, and 1A13P (exon cluster 2), while the polypeptides within an exon cluster are 75–92% identical in sequence (297). This organization most likely evolved from two distinct rounds of duplication and divergence involving the first exons and their flanking sequences. The earliest round led to the generation of four exons, including ancestral first exons of 1A1, 1A6 and the ancestors to the 1A2P-1A5 and 1A7-1A13P exon clusters. A subsequent amplification and divergence event led to formation of the 1A2P-1A5 and 1A7-1A13P exon clusters (294, 297). The UGT1 locus contains numerous remnants of UGT exon sequences throughout the locus, indicating the occurrence of many gene duplication, deletion and conversion events throughout the evolution of the locus; similar to what has been described for several of the CYP450 subfamily clusters of genes (298).

**UGT2 family**

The human UGT2 gene family is located on chromosome 4q13 and includes three members of the UGT2A subfamily and 12 members (seven genes and five pseudogenes) of the UGT2B subfamily (Figure 2-3).
The members in each UGT2 subfamily are >70% similar in sequence (297). The UGT2 genes have been sequentially named based on the chronological order of the discovery of the genes (294). The UGT2 genes each contain six exons that are not shared between the UGT2 family members, except for UGT2A1 and 2A2, which share common exons 2-6 resulting in different N-terminal sequences and an identical 288-amino-acid C-terminal sequence that arises by the differential splicing of a variable first exon to the same set of five downstream exons (297) similar to the UGT1A genes. Exons 1 and 2 of each member of the UGT2 family correspond to exon 1 of each UGT1 family member in terms of the length of translated protein. Similar to the organization of many CYP gene subfamily clusters (298), many gene remnants are present in the UGT2 locus and the UGT2A and the UGT2B genes are intermingled along this stretch of chromosome (Figure 2-3). The C-terminal domains of the UGT1 and UGT2 families are highly homologous, representing conservation of a common UDP-glucuronic acid binding site (295).
**UGT3 family**

The two members of the human UGT3 family have been identified, UGT3A1 and 3A2, and are located on chromosome 5p13.2 (299-301). They consist of seven exons and are separated by approximately 76 kb. They encode proteins of 523 residues, with UGT features such as a putative N-terminal signal peptide, the ‘UGT signature sequence’, a C-terminal transmembrane region, and the dilysine motif (two lysines at positions –3/-4 or –3/-5 from the C-terminus) (294). The UGT3 family was recently identified via sequencing of the human, rat and mouse genomes (301). Members of the UGT3 family share approximately 30% sequence similarity to the UGT1, UGT2 and UGT8 families. The catalytic and physiological functions of UGT3 family members, and their distribution in cells and organs, have been characterized partially. UGT3A1 preferentially catalyzes the transfer of N-acetylglucosamine from UDP N-acetylglucosamine to the bile acid ursodeoxycholic acid (3α, 7β-dihydroxy-5β-cholanoic acid). Additionally, UGT3A1 has activity toward 17α-estradiol, 17β-estradiol, 4-nitrophenol and 1-naphthol (302). UGT3A1 is found in the liver and kidney, and to a lesser extent, in the gastrointestinal tract (302). UGT3A2 uses both UDP-glucose and UDP-xylose to glycosylate a broad range of substrates including 4-methylumbelliferone, 1-hydroxypyrene, bioflavones, and estrogens. It has low activity toward bile acids and androgens. UGT3A2 transcripts are found in the thymus, testis, and kidney but are barely detectable in the liver and gastrointestinal tract (299).

**UGT8 family**

The UGT8 family consists of a single gene in humans that encodes the UDP-galactose ceramide galactosyltransferase enzyme (CGT) (303, 304). The gene consists of five protein-coding exons on human chromosome 4q26. CGT catalyzes the key enzymatic step in the
biosynthesis of galactocerebrosides, by transferring a galactose to ceramide. Galactocerebrosides are abundant sphingolipids of the myelin membrane of the central nervous system and peripheral nervous system and are also present in small amounts in kidney.

**Transcriptional Control and Tissue-Specific Expression**

The UDP glucuronosyltransferases (UGT) are expressed predominantly in the liver and gastrointestinal tract in humans, but are expressed in almost every human tissue (305, 306). Expression of specific UGT genes differs significantly between tissues and individuals and tissue-specific regulation of UGT-gene expression, promoter region polymorphisms, copy number variations, and splice variants are all thought to contribute to inter-tissue and inter-individual expression differences.

Transcription factors account for much of the variability in UGT expression. For example, hepatocyte nuclear factor 1a (HNF1a) regulates several UGT1A and 2B genes by binding to their proximal promoters and enhancing transcription (307-314). Regulation by HNF1a is influenced by other transcription factors. For example, the capacity of HNF1a to activate the UGT2B7 promoter in liver cells is enhanced by octamer transcription factor 1 (Oct-1) tethering to HNF1a and subsequent interaction of Oct-1 with the basal transcription factor (TF) IIB (314). This enhancement of HNF1a-mediated activation is not observed with the UGT2B17 promoter (312), most likely due to differences in the position and orientation of bound HNF1a to the transcription machinery in the UGT2B17 promoter compared to the UGT2B7 promoter.

Furthermore, the UGT2B17 promoter contains a Pre-B cell homeobox transcription factor (Pbx)-binding site which abuts the HNF1 site and, when Pbx-2 and its dimerization partner, Prep1, are bound, HNF1a binding and transcriptional activation is interrupted (311). In the gastrointestinal tract, HNF1a binds to a site conserved in all the promoters of the UGT1A7–1A10 cluster, about
90 bp upstream from the transcription start site. Binding of HNF1a activates the promoters of these genes in Caco-2 cells (315). Downstream from the HNF1 site is a binding site for the intestine-specific transcription factor, caudal-related homeodomain protein 2 (Cdx-2). This site is present in the promoters of UGT1A8, 1A9 and 1A10 but is absent in the UGT1A7 promoter. When Cdx-2 binds to this site, it can independently and differentially activate the UGT1A8 and 1A10 promoters or act synergistically with HNF1a to further activate these promoters. In contrast, activation of the UGT1A9 promoter by Cdx-2 only occurs in cooperation with HNF1a(315). Ligand-activated transcription factors, such as aryl-hydrocarbon receptors (AhRs), hypoxia-inducible factor (HIF), and estrogen response elements (EREs), regulate UGT expression based on cellular conditions (316). Thus, UGT expression is controlled in part by transcription factor binding, variation in 5’ UTR sequences, tissue-specific expression and binding of activators or repressors, and in response to endogenous and exogenous compounds.

Non-coding region polymorphisms impact UGT transcription when they are in regulatory regions in UGT gene promoters. For example, insertion of a TA repeat into the TATA box of the UGT1A1 promoter reduces UGT1A1 promoter activity by about 30% and subsequent UGT1A1 enzyme content of the liver (317). Individuals homozygous for the promoter with 7 TA repeats, A(TA)\textsubscript{7}TAA, (UGT1A1*28) instead of the usual A(TA)\textsubscript{6}TAA (UGT1A1*1) and have an inherited form of hyperbilirubinemia called Gilbert’s syndrome, which is present in about 6–12% of the population (318, 319). The A(TA)\textsubscript{5}TAA (UGT1A1*33) and A(TA)\textsubscript{8}TAA (UGT1A1*34) alleles are rare variants present in African populations that result in a similar phenotype (320). The prevalence of the UGT1A1*28 polymorphism varies significantly with ethnicity, being present in 23% of Africans, but less than 3% of Asians. Gilbert’s syndrome is characterized by slightly elevated serum bilirubin levels basally, which become more pronounced under conditions of enhanced hemolysis.
Transcription factor polymorphisms can affect UGT transcription as well. For example, HNF1a variants were tested for their capacity to enhance the activity of the UGT2B17 promoter in HepG2 cells. HNF1a activated the UGT2B17 promoter about 20-fold. This activation was significantly decreased by about one-third with the L27 and N487 HNF1a genetic variants and was completely abolished with the HNF1a protein containing the P291fsinC frameshift mutation (321). Copy number variations, including gene duplications and deletions, of individual UGT genes have been described as gene polymorphisms that contribute to inter-ethnic differences and tissue-specific expression of UGTs as well (322-324).

Finally, splice variants of the UGTs have been reported for all functional UGT1A family members and for UGT2B4 (325-327). These alternately spliced variants have a novel or extra exon at their 3'-end that renders them functionally inactive. These variants interact with classic UGT isoforms forming dimers that repress UGT-mediated reactions by up to 80% (327). Tissue-specific expression of these variants is similar to the expression of the classic UGT isoforms and expression of each variant varies by tissue, likely controlled by tissue-specific transcription factors.

Translation and Post-Translational Modification

UGT mRNA is translated into protein by the ribosome in the rough endoplasmic reticulum (ER). Phosphorylation of specific translational machinery, such as eukaryotic initiation factors (eIFs) can regulate translation, as can microRNA. Very little work has been done in the field of translational regulation of UGTs. UGTs undergo a number of post-translational modifications, including N-glycosylation (328) and phosphorylation (329).
UGTs are synthesized as precursor proteins of about 530 residues that contain an N-terminal signal peptide that mediates the targeting and integration of the polypeptide chain from the cytoplasm into the ER (330-332) with the assistance of the signal recognition particle (SRP) and its membrane receptor (333). The polypeptide is then translocated into the ER by a multiprotein assembly complex called the translocon (334). The translocon provides an aqueous protein conducting channel spanning the membrane bilayer. The translocon is able to bind ribosomes with high affinity, recognize functional signal sequences, and allow the lateral partitioning of transmembrane domains (TMDs) into the lipid bilayer. The orientation and integration of membrane proteins determine protein topology and are coupled to protein folding. Many prediction algorithms have been developed in order to determine the topology of integral membrane proteins (335).

UGTs are predicted to be type I ER membrane proteins containing an N-terminal signal peptide that is cleaved, a glycosylated luminal domain, and a short cytoplasmic tail. The majority of the protein is located in the ER lumen and attached to the membrane via a C-terminal TMD (336). The mature protein is approximately 505 residues (337, 338). Members of the UGT1A family share an identical C-terminus (~245 amino acids) containing the common cosubstrate UDP-glucuronic acid binding site, whereas the N-terminal region (~286 amino acids) shows a markedly lower level of identity (37–49%). The N-terminal portion of the enzyme contains the substrate binding site and includes two hypervariable regions (339). UGT2 family enzymes do not share a common C-terminal domain except for the UGT2A1 and UGT2A2 genes (297). Comparison of the members of the UGT2 gene family indicates that amino acid differences between different isoforms occur throughout the length of the protein, although the C-terminal
halves are highly conserved. Based on protein sequence analysis of UGT isoforms, it is generally accepted that the variable N-terminal domain binds substrate and therefore determines the substrate specificity, whereas the C-terminal domain binds the common substrate, UDP-glucuronic acid (340).

UGTs undergo protein-protein interactions that can alter their function. Homo- and hetero-UGT interactions have been reported (341, 342), as have interactions between UGTs and CYP450 enzymes (343, 344). UGTs isolated from rat coeluted with rat CYP1A1 using a bovine serum albumin-conjugated Sepharose 4B column (345). Using a coimmunoprecipitation method, UGT2B7, UGT1A6, UGT1A1, and CYP3A4 were immunoprecipitated with specific antibodies for each enzyme (346). Coimmunoprecipitation of P4503A4 with UGT2B7 has been reported (343). These data suggest that UGT isoforms may form complexes with each other, and UGT isoforms may interact with CYP450s. Hetero-oligomers between UGT1A isoforms and UGT2B1 have been reported in HLM (347). This complex was copurified using specific Sepharose-conjugated antibodies directed against either UGT1A or UGT2B1 isoforms and confirmed by crosslinking experiments. Interactions between CYP450 and UGT are mainly confined to the transmembrane (TM) domains, although the interaction between the short N-terminal tail of the CYP450 and catalytic domain of UGT, or the short C-terminal tail of UGT and catalytic domain of CYP450 may also occur (295). Protein–protein interactions may modify the activity and substrate specificity of the enzyme (295).

**Cellular Location**

UGTs are type I endoplasmic reticulum (ER) membrane proteins with a glycosylated luminal (amino-terminus) domain (336) (Figure 2-4).
UGTs that catalyze the biosynthesis of oligosaccharides such as heparin or chondroitin sulfates have been reported to associate with the Golgi apparatus (348-350). UGT1A6 and UGT2B7 have been shown in the inner and outer nuclear membranes of human liver, suggesting a possible role of these UGTs as a protective barrier against substances toxic to the genome (351, 352). No cytosolic form of UGT has been described in mammals and, although UGTs have been reported in the mitochondria or plasma membranes during subcellular fractionalization, these data are controversial (353, 354).

It is believed that all the UGTs have a similar topology in the ER membrane (Figure 2-4). The luminal domain consists of about 470-480 amino acid residues from the amino terminal (95%) and includes the catalytic site (352, 355). The cytoplasmic domain contains about 19-26 amino acid residues (55) and the carboxy terminus (337, 338). These two domains are connected through the ER lipid bilayer by a transmembrane domain conserved between UGTs consisting of 17 hydrophobic residues between a N-terminal aspartate and a C-terminal lysine (337, 356). The transmembrane domain acts as an ER retention domain along with a dilysine motif (KSKTH) positioned at amino acids 3 and 5 from the carboxy-tail, together acting as static and dynamic ER retention signals, respectively (336, 357, 358). The transmembrane domain acts as a halt signal anchoring the UGT polypeptide in the ER membrane. UGTs are directed from the cytoplasm to the ER via an amino-terminal signal peptide, which is cleaved to yield the mature enzyme (336).
UDP-Glucuronosyltransferase (UGT) Enzymes are part of phase II (conjugation) metabolism and covalently link glycosyl groups (i.e. glucose, glucuronic acid, xylose, galactose, etc.) to a functional group (-OH, -SH, -NH2, C-C, or -COOH) of a xenobiotic or endobiotic lipophilic substrate (295). UGT-Enzyme-catalyzed glucuronidation reactions total about 35% of phase II reactions (23) and, along with CYP450 Enzyme-catalyzed reactions, account for the majority of xenobiotic metabolism. UGT Enzymes have broad, overlapping substrate specificities (359-361) likely due to a highly pliant active site. As the active sites of CYP450 Enzymes face the ER membrane, UGT Enzymes may preferentially accept hydroxylated substrates from the ER membrane (362). In general, conjugation reactions result in the formation of water-soluble, pharmacologically inactive metabolites. However, some biologically active or highly reactive conjugated metabolites can also be generated. For example, morphine-6-O-glucuronide has greater analgesic effects in suppressing pain symptoms in humans than its parent compound morphine (363-366).

Mammal UGTs are able to use nine different UDP-glycosyl donors, but UGTs within a given family show preference for certain UDP-sugars. UGT1 and UGT2 enzyme families utilize UDP-glucuronic acid as the glycosyl donor most efficiently (367, 368); UGT3A1 utilizes UDP-N-acetylglucosamine preferentially (300) while UGT3A2 utilizes UDP glucose and UDP xylose (299), and UGT8A1 utilizes UDP-galactose as the sugar donor (369). The UGTs of other organisms, including plants, use UDP-glucose predominantly as the glycosyl donor (370). UDP-glucuronic acid is made from UDP-glucose in a reaction catalyzed by uridine diphosphate glucose dehydrogenase, an oxoreductase enzyme that participated in 4 mammalian metabolic pathways: pentose and glucuronate interconversions, ascorbate and aldarate metabolism, starch and sucrose metabolism, and nucleotide sugars metabolism (371).
The UDP-sugar co-substrates must be transported from the cytosol to the catalytic site of the UGT enzyme in the luminal side of the ER, which is facilitated by UDP-galactose transporter-related isozyme7 (372-374). This translocation is a rate-limiting step in many glucuronidation reactions (375-377). UGT glucuronide products formed in the ER lumen are rapidly translocated to the cytosol by a yet unidentified transporter. Finally, glucuronides are transported from the cytosol, across the plasma membrane via a MDR protein family 1, 2, or 3 (378, 379) or organic anion 2, 4, or 8 transporters (380-382) into the bile or blood.

**UGT Enzymes and the Severe Weight Gain-Inducing SGAs**

The SGAs that induce “severe weight-gain” include CLZ and OLZ (156, 160, 161). Weight gain-induced by both OLZ and CLZ appears to be proportional to plasma concentrations of the drugs (19, 20, 246), especially in studies of antipsychotic-naïve individuals. CLZ plasma levels between 350-600 ng/mL are efficacious with a low incidence of adverse effects (48, 383-387); however plasma levels between 3-1,504 ng/mL have been reported in patients administered the same dose (19, 31, 388) and plasma levels do not necessarily correlate with the dose administered (17, 19, 223). Although it is suggested that the increased efficacy of CLZ is due in part to dmCLZ activity, the metabolism of this active metabolite has not been extensively studied and little is known regarding how DMEs mediate its clearance and inactivation. Plasma concentrations outside of the suggested therapeutic range increase a patient’s risk for weight gain or lack of efficacy (42, 387, 389, 390). This also occurs with the severe weight gain-inducing OLZ (20, 391), for which ideal plasma concentrations have been defined as 20-80 ng/ml (392, 393) but large inter-individual variability in plasma concentrations have been reported (36, 394).
UGTs are an important component of the biotransformation and clearance of the severe weight gain-inducing SGAs as evidenced by the percentage drug-glucuronides contribute to total metabolites in patient plasma, urine, and feces (25-27). 97% of administered CLZ is biotransformed (17) and ADME studies in humans have identified two primary CLZ-glucuronides and at least five secondary glucuronides (26-28). Three of these metabolites account for approximately 30% of human CLZ metabolites (26); the rest have not yet been quantified (27). One of these reported secondary glucuronides is of the abundant, active metabolite dmCLZ (28), which is involved in eliciting agranulocytosis in 1-2% of patients taking CLZ (29). It is unclear whether glucuronidation is a major or minor metabolic pathway of dmCLZ but glucuronidation may be an important clearance mechanism for dmCLZ and the reactive metabolite of dmCLZ involved in development of agranulocytosis.

UGT activity against CLZ has been partially characterized: in vitro, UGT1A4 forms tertiary and quaternary CLZ-N-glucuronide metabolites, and the UGT1A4<sup>L48V</sup> polymorphism was twice as efficient as wild-type UGT1A4 (32). This, along with the extensive role of UGT enzymes in the metabolism and clearance of CLZ (17, 19, 26), suggests that UGT genetic variants likely influence CLZ plasma levels. But, for CLZ and many SGAs, the UGTs with drug-specific enzymatic activity have not been or have been only partially characterized. Likewise, the effects of many UGT enzyme polymorphisms have not been examined in vitro or in vivo.

Glucuronidation is the major mode of OLZ metabolism, accounting for 25% of the administered dose. Three major glucuronide metabolites are made in humans: OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, and OLZ-4’-N-glucuronide. Previous in vitro studies using over-expressing baculosomes demonstrated that UGT1A4 is active against OLZ, with no activity observed for UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B7. However, kinetic data for OLZ glucuronidation could not be established for human liver microsomes (HLM) using OLZ concentrations spanning the apparent K<sub>M</sub> of UGT1A4, suggesting that other untested UGT
enzymes contribute to OLZ glucuronidation *in vivo* (33). There is large interindividual variability in OLZ clearance (20, 21, 34-37) and studies indicate clinical outcomes and plasma concentrations are related (21). Weight gain and obesity are seen in a subset of patients taking OLZ (38) and the evidence suggests that there is a dose-response relationship between OLZ serum concentrations and metabolic outcomes (20, 39-42, 390, 391, 395). Recently the UGT1A4 *3 allele (142 T>G nucleotide change, L48V amino acid change) was shown to be a significant predictor of OLZ plasma levels, resulting in a 5-fold reduction compared to the wild-type allele (36). Figure 2-5 outlines the enzymes previously shown to be involved in OLZ metabolism and the relative urinary contribution of each pathway (396). In subjects taking OLZ, unchanged OLZ comprises ~18% of total urinary OLZ; OLZ glucuronides comprise nearly 50% of all urinary OLZ metabolites, with the 10-N-glucuronide comprising ~86% of urinary OLZ glucuronides (254, 397, 398).
Schematic of OLZ metabolism. Previous studies identified the 10-N-glucuronide as the major metabolite of OLZ in urine, feces, and plasma (205). Percentages are reported OLZ metabolite recovery in urine (206). Enzymes responsible for the metabolism of OLZ are indicated, with the most active enzymes indicated with a larger font.
Chapter 3

SCREENING AND KINETICS OF UDP-GLUCURONOSYLTRANSFERASE ENZYMES WITH ACTIVITY AGAINST SEVERE WEIGHT GAIN-INDUCING SECOND-GENERATION ANTIPSYCHOTICS

This chapter outlines the methods, results, and implications of a thorough characterization of the glucuronidation pathway involved in the metabolism of the severe weight gain-inducing SGAs OLZ, CLZ, and its active metabolite dmCLZ. The studies in this chapter include an initial characterization of OLZ, CLZ, or dmCLZ glucuronidation based on HLM, screening of the 14 UGT1A and 2B enzymes known to be involved in xenobiotic metabolism using over-expressing cellular homogenates, and a characterization of wild-type UGT kinetic activity against OLZ, CLZ, and dmCLZ.

Introduction

The SGAs OLZ and CLZ are FDA (U.S.)-approved for use in schizophrenia (55), bipolar disorder (399, 400), and treatment-resistant depression in combination with fluoxetine (55), and refractory schizophrenia, respectively. Both OLZ and CLZ improve schizophrenic negative symptoms such as conceptual organization, social interaction, and mood while decreasing positive symptoms such as delusions and paranoia (401) with a lower incidence of extrapyramidal side effects, less prolactin elevation, and improved patient compliance compared to FGAs (402, 403). Although OLZ has a lower discontinuation rate and a greater reduction in psychopathology, (140, 404), it is associated with severe weight gain in patients (404-409), contributing to increased metabolic dysfunction, dyslipidemia, overweight/obesity, type II diabetes mellitus, heart disease, and mortality, as is CLZ (410, 411). Furthermore, CLZ is associated with a 1% risk
of agranulocytosis, thought to be mediated by reactive intermediates of CLZ and its active and abundant metabolite, dmCLZ, interfering with leukocyte formation (412-415). While not FDA approved for treatment, dmCLZ demonstrates similar receptor binding activity as CLZ at 5-HT\textsubscript{2C} and D\textsubscript{2} receptors and has greater binding affinity at M\textsubscript{1} receptors, which may mediate a greater antipsychotic effect due to interactions with NMDA receptors and the glutaminergic system (416). Thus, dmCLZ has therapeutic potential that is only shadowed by its association with agranulocytosis (417).

OLZ, usually administered to patients orally at a dose of 8 - 64 µg a day, is excreted predominantly in urine by the enzymatic addition of glucuronic acid by the UGT family of phase II metabolizing enzymes (35, 254). Inhibition of OLZ glucuronidation in patients by co-administering OLZ with probenecid resulted in elevated plasma levels of OLZ compared to OLZ administered alone (197). Published studies have shown that OLZ is extensively metabolized. The OLZ-10-N-glucuronide is the major metabolic product in human plasma and urine, the only metabolite identified in feces, accounting for 25% of the overall OLZ dose (35, 254, 398). Other OLZ glucuronide products have been reported in humans, including the directly conjugated OLZ-4’-N-glucuronide (35, 254, 398). In addition to increasing hydrophobicity and excretion of OLZ and its metabolites, glucuronidation deactivates the compound as indicated by decreased binding to known OLZ receptors (397). Previous \textit{in vitro} studies using over-expressing baculosomes demonstrated that UGT1A4 is active against OLZ, with no activity observed for UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B7. However, kinetic data for OLZ glucuronidation could not be established for human liver microsomes (HLM) using OLZ concentrations spanning the apparent K\textsubscript{M} of UGT1A4, suggesting that other untested UGT enzymes may also contribute to OLZ glucuronidation \textit{in vivo} (33). While UGT1A4 had been shown to be active against OLZ, several UGTs have not previously been screened. The goal of the present study was to characterize the glucuronidation activity of known UGT1A and 2B family enzymes against OLZ \textit{in vitro}.
Materials and Methods

Chemicals and Materials

OLZ was purchased from Toronto Chemicals (Toronto, Canada). CLZ, dmCLZ, alamethicin, β-glucuronidase, bovine serum albumin, anti-calnexin antibody, and lamotrigine (LTG) were purchased from Sigma-Aldrich (St. Louis, MO). DMEM, Dulbecco’s PBS (minus calcium chloride and magnesium chloride), fetal bovine serum, penicillin-streptomycin, geneticin (G418), Platinum Pfx DNA polymerase, and the pcDNA3.1/V5-His-TOPO mammalian expression vector were all obtained from Invitrogen (Carlsbad, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL). The human anti-UGT1A polyclonal antibody and standard were purchased from Gentest (Woburn, MA). Anti-β-actin monoclonal antibody was obtained from Sigma. Polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies (Coralville, IA). All other chemicals were purchased from Fisher Scientific (Waltham, MA) unless specified otherwise.

Tissues

The normal human liver tissue specimens used for these studies have been described previously (418, 419). Normal adjacent liver specimens were obtained from patients and quick-frozen at -80°C within 2 h post surgery. HLM were prepared through differential centrifugation as previously described (420) and stored (10–20 mg microsomal protein/mL) at -80°C. Microsomal protein concentrations were measured using the BCA assay. All protocols involving the analysis of tissue specimens were approved by the institutional review board at the Penn State College of Medicine and in accordance with assurances filed with and approved by the United States Department of Health and Human Services.
Cell Lines

The cell lines over-expressing the UGT1A and UGT2B isoforms used in this study were described previously (421-426). All UGT over-expressing cell lines were grown in DMEM to 80% confluence before preparing cell homogenates by resuspending pelleted cells in Tris-buffered saline (25 mM Tris base, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) and subjecting them to three rounds of freeze-thaw before gentle homogenization. Total homogenate protein concentrations were measured using the BCA protein assay. Homogenates were stored at -80°C in 200-µL aliquots to minimize freeze-thaws.

Western Blot Analysis

UGT1A4 protein levels were determined by Western blot analysis as described previously using the UGT1A antibody purchased from Gentest (Woburn, MA) (425, 426). UGT1A4 protein levels were quantified against 100-250 ng of human UGT1A protein (Gentest) by densitometric analysis of X-ray film exposures (5 s to 2 min exposures) of Western blots using a GS-800 densitometer with Quantity One software (Bio-Rad, Hercules, CA). Quantification was made relative to the levels of calnexin observed in each lane. Antibodies were used at a 1:5000 dilution. For UGT1A4, relative protein levels were expressed as the mean of three independent Western blot experiments and all activity assays were normalized relative to UGT1A4 expression in the UGT1A4-over-expressing cell line. 250-400 µg of UGT2B10- or UGT2B10 variant (51.8 kDa)-over-expressing cell lines along with 250 µg HEK 293 and 335 ng UGT2B7 (60.7 kDa) standard protein (BD Gentest, Woburn, MA) were adjusted to contain equal volumes of loading buffer and heated at 100°C for 10 min. Samples were run at 93 V on a 10% acrylamide gel then transferred to a Polyvinylidene difluoride (PVDF) membrane for 2 h at 30 V. PVDF membranes were then probed with goat polyclonal UGT2B antibody purchased from Santa Cruz Biotechnology, Inc. (1:500 dilution, Santa Cruz, CA) for 1 h at 23°C, washed three times, followed by horseradish
peroxidase-conjugated donkey anti-goat IgG (1:4500 dilution, Santa Cruz, CA). UGT2B protein was visualized using the SuperSignal West Dura Extended Duration Substrate from ThermoScientific and Hyblot CL Autoradiography film (Deville Scientific, Metuchen, NJ). UGT2B10 protein levels were relatively quantified against β-actin by densitometric analysis of X-ray film exposures (2 to 4 min exposures) of Western blots using ImageJ software (U.S. National Institutes of Health, Bethesda, MD). B-actin was probed using an anti-β-actin antibody at 1:2000 dilution on the once stripped western. Similar to that described above for UGT1A4, all UGT2B10 activity assays were normalized relative to UGT2B10 expression in the UGT2B10-over-expressing cell line.

Glucuronidation Assays

Homogenates and HLM were incubated with alamethicin (50 μg/mg protein) for 15 min on ice similar to that described previously (425, 427). Glucuronidation assays were performed in 50 mmol/L Tris buffer (pH 7.4), 10 mmol/L MgCl$_2$, 4 mmol/L UDP-glucuronic acid (UDPGA), and 7.8 μmol/L to 4 mmol/L of substrate at 37°C in a water bath. Glucuronidation assay incubation times were 30 min for CLZ and dmCLZ UGT1A4 assays, and 2h for CLZ UGT1A1, 1A3, and 2B10, and OLZ UGT1A4 and 2B10 assays. HLM, BLM, and PLM (200 μg of protein) or human UGT-over-expressing cell homogenate (250 μg of protein for OLZ, 1 mg for CLZ and dmCLZ) were screened for glucuronidation activity using 50 μM, 160 and 306 μM, and 160 and 320 μM of OLZ, CLZ or dmCLZ respectively in a 50 μL reaction. Kinetic assays were performed in 20 or 50 μL reactions using a range of 9.4-2000 μM of OLZ and 50 μg and 1.5 mg of UGT1A4- and UGT2B10-over-expressing cell homogenate protein, respectively, or 12.5 μg of HLM protein for OLZ. 10-612 μM of CLZ and 250 μg, 1 mg, 50 μg, and 1.5mg of UGT1A1-, UGT1A3-, UGT1A4-, or UGT2B10-over-expressing cell homogenate protein, respectively, or 12.5 μg of HLM protein were used for CLZ kinetic assays, and 10-640 μM of dmCLZ and 50 μg
UGT1A4-over-expressing cell homogenate protein or 12.5 μg of HLM protein were used for dmCLZ kinetic assays. Reactions were terminated by the addition of the same volume of cold acetonitrile as the initial reaction volume. Reactions were centrifuged at 13,000 g for 10 min at 4°C and supernatants were collected. Glucuronidation assays (5 μL) were analyzed for drug-glucuronide formation using a Waters ACQUITY ultra pressure liquid chromatography (UPLC) system (Milford, MA) as previously described (418, 428) using a 100 x 2.1 mm inner diameter Acquity UPLC ethylene bridged hybrid (BEH) C18 column with 1.7 μm particles (Waters) and a 0.2 μm prefilter installed before the column. Elution for OLZ consisted of a gradient elution starting with 17.5% buffer B (100% acetonitrile) and 82.5% buffer A [5mmol/L ammonium acetate (pH 6.0)] for 5 min, a linear gradient to 90% buffer B over 1 min and held for 2 min, then a linear gradient back to initial conditions and held for 2 min for a total run time of 10 min. The flow rate was maintained at 0.5 mL/min. Elution for CLZ and dmCLZ consisted of a gradient elution starting with 10% buffer B (100% acetonitrile) and 90% buffer A [20mmol/L ammonium acetate (pH 7.0)] for 2 min, a linear gradient to 30% buffer B over 1 min and held for 1 min, a linear gradient to 75% buffer B over 2 min and held for 2 min then a linear gradient back to initial conditions for 1 min and held for 1 min for a total run time of 10 min. The flow rate was maintained at 0.5 mL/min. The amount of glucuronide formed was determined based on the ratio of drug-glucuronide versus unconjugated drug after calculating the area under the curve for the drug and drug-glucuronide peaks using the known amount of drug added to each reaction as the reference. drug-glucuronide was confirmed by sensitivity to treatment with 1,000 U β-glucuronidase at 37°C for 12-16h as previously described (419), by treatment with 3 N HCl at 50°C for 1 h (254), and by mass spectrometry (described below). As controls, glucuronidation assays were performed using HLM as a positive control for glucuronidation activity and untransfected HEK293 cell homogenate protein which served as a negative control for
glucuronidation activity. Two independent experiments were performed for UGT screening of over-expressing cell homogenates.

**Mass Spectrometry**

Triple-quadrupole tandem mass spectrometric detection was performed using an ACQUITY SQD (Waters Corp.) with electrospray ionization interface and an UPLC system consisting of a binary gradient pump, an auto sampler (4°C), and a column oven (40°C). UPLC was operated under the same conditions as described above for glucuronidation assays. Peaks were detected at 270 nm wavelength for OLZ and 264 nm for CLZ and dmCLZ. The mass spectrometer operated in positive mode was set up to scan the daughter ion of m/z 312.43 for OLZ, m/z of 327, 503, and 679 for CLZ, and an m/z of 313 and 489 for dmCLZ. The optimized mass spectrometry parameters used were as follows: capillary voltage, 0.57 kV; cone voltage, 30 V; collision energy, 15 V; source temperature, 450°C; and desolvation temperature, 140°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 760 L/h. Argon was used as the collision gas at a flow rate of 0.1 mL/min. Data acquisition and analysis were performed using the MassLynx NT 4.1 software with QuanLynx program (Waters Corp.).

**Statistical Analysis**

Michaelis-Menten kinetic constants were determined using Prism Version 5 software (La Jolla, CA). The two-sample t-test (two-tailed) was used to compare kinetic values of glucuronide formation for the different UGT isoforms in cell lines and HLMs.
Results

Characterization of OLZ Glucuronidation

UGT Screening

To better characterize the enzymes responsible for hepatic OLZ glucuronidation, glucuronidation assays were performed and OLZ-glucuronides were separated by UPLC. In addition to an OLZ peak at a retention time of 6.2 min (Figure 3-1, panel B), incubations of OLZ with HLM yielded three peaks at 1.45 min, 1.78 min, and 3.83 min (Figure 3-1, panel C). While the 3.83 min peak was highly sensitive to treatment with β-glucuronidase (Figure 3-1, D), the peaks at 1.45 and 1.78 min were less sensitive to this treatment but were extremely sensitive to treatment with 3N HCl solution, a pattern that was previously reported for these OLZ glucuronides (254, 398).
The peaks at 1.45, 1.78, and 3.83 min all demonstrated a [M'] peak at $m/z$ 489 (the glucuronide conjugate of OLZ) by mass spectrometry (MS/MS) analysis. The peaks at 1.45 and 1.78 min showed a [M+H]$^+$ peak at $m/z$ 313 for OLZ after loss of the glucuronic acid moiety (molecular weight = 176 g/mol) and a $m/z$ 432 fragment after loss of the CH$_2$=CH-NH-CH$_3$ methyl piperazine moiety (Figure 3-2, panel A).
Figure 3-2 MS/MS analysis of OLZ glucuronides formed by HLM

Panel A, mass spectra of UPLC peaks 1 and 2; panel B, mass spectra of peak 3; Peak 1, OLZ-10-N-glucuronide isomer 1; peak 2, OLZ-10-N-glucuronide isomer 2; peak 3, OLZ-4'-N-glucuronide; peak 4, OLZ; peak 5, UDP-glucuronic acid. AU, absorbance.
This is identical to the pattern observed for the two OLZ-10-\(N\)-glucuronide isomers reported previously (254, 398). The peak at 3.83 min (Figure 3-2, panel B) demonstrated a \([\text{M+H}]^+\) peak at \(m/z\) 313 (OLZ) and at \(m/z\) 282 due to the loss of \(\text{CH}_3\text{NH}_2\) and glucuronic acid, but no peak at \(m/z\) 423, which is characteristic of the OLZ-4'\(<\text{-N}\)-glucuronide (254, 398).

Previous studies of selected UGT enzymes suggested that UGT1A4 was active against OLZ. To fully characterize all of the UGTs responsible for OLZ glucuronidation, a comprehensive screening of OLZ glucuronidation activity by homogenates from HEK293 cells over-expressing wild-type UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17 was performed. Two UGTs exhibited detectable levels of activity against OLZ: the hepatic UGTs 1A4 and 2B10. None of the other UGTs screened in our assays (UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B11, 2B15 or 2B17) exhibited any glucuronidation activity against OLZ using up to 250 \(\mu\)g of UGT-over-expressing cell homogenate. The UPLC 3-peak pattern and retention times for UGTs 1A4 and 2B10 were identical to that observed for HLM and showed similar sensitivity to treatment with \(\beta\)-glucuronidase and 3N HCl solution. Similar to that observed for OLZ glucuronides observed in incubations with HLM, the UGT1A4- and UGT2B10-generated peaks were identified as OLZ-10-\(N\)-glucuronide isomer 1 at 1.45 min, OLZ-10-\(N\)-glucuronide isomer 2 at 1.78 min, and OLZ-4’-\(N\)-glucuronide at 3.83 min as determined by UPLC/MS/MS analysis (Figure 3-3).
Figure 3-3 UPLC analysis of OLZ glucuronides formed by HLM, UGT 1A4 and 2B10

Glucuronidation assays were performed using 12.5 µg HLM, 50 µg UGT1A4, and 1.5 µg UGT2B10 protein and 300 µM OLZ, and incubated at 37°C for 2 h with 4 mM UDP-glucuronic acid prior to analysis by UPLC as described in the Materials and Methods. Panel A, HLM + vehicle (1% DMSO); panel B, UGT-null HEK 293 cell homogenates + OLZ; panel C, HLM + OLZ; panel D, HLM + OLZ and 1,000 U of β-glucuronidase; panel E, UGT1A4 + OLZ; panel F, UGT2B10 + OLZ. OLZ-10-N-glucuronide isomer 1; peak 2, OLZ-10-N-glucuronide isomer 2; peak 3, OLZ 4'-N-glucuronide; peak 4, OLZ; peak 5, UDP-glucuronic acid. AU, absorbance.
**UGT Kinetics**

Representative kinetic plots of glucuronidation rate versus substrate concentration are shown in Figure 3-4 for wild-type UGTs 1A4 (UGT1A4$^{24Pro^{48Leu}}$, top panels) and 2B10 (UGT2B10$^{67Asp}$; middle panels) as well as HLMs with wild-type UGTs 1A4 and 2B10 (bottom panels).

**Figure 3-4 Kinetic curves for wild-type UGT1A4$^{24Pro^{48Leu}}$- and UGT2B10$^{67Asp}$-over-expressing cell homogenates and wild-type HLM against OLZ**

Representative kinetic curves were performed as described in the Materials and Methods using OLZ concentrations of 9.4, 18.8, 37.6, 75, 150, 300, 400, 600, 800, 1000, 1200, 1600, and 2000 μM for cell lines and 62.5, 125, 250, 500, 1000, and 2000 μM for HLM. Wild-type HLM were from subjects exhibiting the UGT1A4(*1/*1)/UGT2B10(*1/*1) genotype.
UGT2B10 exhibited a significantly ($p<0.0001$) decreased binding affinity against OLZ ($K_M = 564 \pm 15 \, \mu M$) as compared to UGT1A4 ($K_M = 156 \pm 17 \, \mu M$) for the formation of OLZ-10-$N$-glucuronide isomer 1 and for OLZ-10-$N$-glucuronide isomer 2 ($K_M = 818 \pm 23 \, \mu M$ vs. 206 $\pm 37 \, \mu M$, respectively; $p<0.0001$; Table 3-1). A significantly ($p=0.0018$) higher binding affinity was observed for UGT2B10 ($K_M = 457 \pm 11 \, \mu M$) as compared to UGT1A4 ($K_M = 810 \pm 135 \, \mu M$) when forming the OLZ-4$'$-$N$-glucuronide. The $V_{\text{max}}$ for formation of OLZ-10-$N$-glucuronide isomer 1 and 2 by UGT2B10 was 12- and 2.3-fold lower than for the reaction catalyzed by UGT1A4 (both $p<0.0001$) and 2.7-fold lower for OLZ-4$'$-$N$-glucuronide formation ($p=0.002$).

Table 3-1 Kinetic analysis of OLZ glucuronidation by UGT-overexpressing cell lines

<table>
<thead>
<tr>
<th>UGT variant</th>
<th>OLZ-10-$N$-glucuronide isomer 1</th>
<th>OLZ-10-$N$-glucuronide isomer 2</th>
<th>OLZ-4$'$-$N$-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (pmol/min/\mu g)</td>
<td>$K_M$ (\mu M)</td>
<td>$V_{\text{max}}/K_M$ (pmol/min/\mu g)</td>
</tr>
<tr>
<td>UGT1A4$^{[\text{38,39}]}$</td>
<td>11 $\pm$ 0.8</td>
<td>156 $\pm$ 17</td>
<td>70 $\pm$ 2</td>
</tr>
<tr>
<td>UGT2B10$^{[\text{38,39}]}$</td>
<td>0.92 $\pm$ 0.02$^b$</td>
<td>564 $\pm$ 15$^c$</td>
<td>2 $\pm$ 0.1$^b$</td>
</tr>
</tbody>
</table>

*For UGT1A4, value represents $K_M$ (\mu M); UGT2B10 protein levels were quantified relative to UGT2B7 protein standard by Western blotting as described in the Materials and Methods and kinetic data are shown as $V_{\text{max}}/K_M$. $^b$ p=0.0001, $^c$ p=0.002 versus corresponding value for UGT1A4$^{[\text{38,39}]}$.

UGT2B10 protein expression in the UGT2B10-over-expressing cell lines was analyzed by Western blot analysis (Figure 3-5). As a UGT2B10 protein standard was not available, the purchased UGT2B7 protein standard (reported to run at 58 kDa; personal communication, BD Gentest technical support) was used to quantify UGT2B10 protein. Western blot analysis demonstrated similar levels of UGT2B10 expression in the UGT2B10 wild-type and codon 67 variant-over-expressing cell lines, which were in agreement with UGT2B10 mRNA levels determined by real-time PCR as previously published for these cell lines (421, 422).
Figure 3-5 Western blot analysis of UGT2B10-overexpressing cell lines

PVDF membrane was probed with 1:500 UGT2B antibody for 1 h at 23°C followed by donkey anti-goat IgG conjugated to horseradish peroxidase (1:4500) for 45 min at 23°C.

Lane 1. HEK 293 (250 µg). Lane 2. UGT2B7 protein standard (335 ng). Lane 3. blank. Lane 4. UGT2B10-over-expressing cell line (250 µg). Lane 5. UGT2B10<sup>677Y</sup>-over-expressing cell line (250 µg). Lane 6. UGT2B10<sup>677Y</sup>-over-expressing cell line (400 µg). Lane 7. UGT2B10-over-expressing cell line (400 µg).

Characterization of CLZ and dmCLZ glucuronidation

**UGT Screening**

Figure 3-6 shows UPLC tracings characterizing the elution pattern of CLZ and its glucuronides.
Figure 3-6 UPLC analysis of CLZ glucuronides formed by HLM.

Glucuronidation assays were performed using 1.25 μg HLM protein and 160 μM CLZ, and incubated at 37°C for 2 h with 4 mM UDP-glucuronic acid prior to analysis by UPLC as described in the Materials and Methods. Panel A. HLM + vehicle (1% DMSO); panel B. UGT-null HEK 293 cell homogenates + CLZ; panel C. HLM + CLZ; panel D. HLM + CLZ after treatment with 1,000 U of β-glucuronidase and 3N HCl for 30 min. Peak 1, CLZ-5-N-glucuronide; peak 2, CLZ-N-glucuronide; peak 3, CLZ; peak 4, UDP-glucuronic acid. AU, absorbance.

CLZ eluted at 6.9 min and, when incubated with HLM, formed two additional peaks eluting at 4.35 and 5.2 min. The 5.2 min peak was highly sensitive to treatment with β-glucuronidase, while the earlier eluting peak at 4.35 min was extremely sensitive to treatment with 3N HCl solution, a pattern that was previously reported for CLZ glucuronides (28). The peaks at 4.35 and 5.2 min both demonstrated a [M⁺] peak at m/z 503 (the glucuronide conjugate of CLZ) by mass spectrometry (MS/MS) analysis (Figure 3-7).
Figure 3-7 MS/MS analysis of CLZ glucuronides formed by HLM

The peak at 4.35 min showed a [M+H]^+ peak at m/z 327 for CLZ after loss of the glucuronic acid moiety (molecular weight = 176 g/mol) and a m/z at 446 and 270 fragment after loss of the CH₂=CH-NH-CH₃ methyl piperazine moiety with and without a glucuronic acid moiety (Figure
3-7, panel A). This is identical to the pattern identified for the CLZ-5-N-glucuronide reported previously (28). The peak at 5.2 min (Figure 3-7, panel B) demonstrated a [M+H]\(^+\) peak at \(m/z\) 327 (CLZ) and at \(m/z\) 472 due to the loss of CH\(_3\)NH\(_2\) and glucuronic acid, but no peak at \(m/z\) 446, which is characteristic of the CLZ-N\(^+\)-glucuronide (27, 28).

dmCLZ eluted at 4.9 min and formed one glucuronide eluting at 2.95 min by UPLC (Figure 3-8).

**Figure 3-8 UPLC analysis of dmCLZ glucuronides formed by HLM**

The peak at 2.95 min was extremely sensitive to treatment with 3N HCl solution, suggesting it is the dmCLZ-5-N-glucuronide previously detected in human urine (28, 429).
Previous studies of selected UGT enzymes suggested that UGT1A3 and 1A4 were active against CLZ and UGT1A4 was active against dmCLZ. To fully characterize all of the UGTs responsible for glucuronidation of CLZ and its active metabolite, a comprehensive screening of CLZ and dmCLZ glucuronidation activity by homogenates from HEK293 cells over-expressing wild-type UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17 was performed. Four UGTs exhibited detectable levels of activity against CLZ using multiple reaction monitoring (MRM): the hepatic UGTs 1A1, 1A3, 1A4 and 2B10. None of the other UGTs screened in our assays (UGTs 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B11, 2B15 or 2B17) exhibited any glucuronidation activity against CLZ using up to 1 mg of UGT-over-expressing cell homogenate. UPLC retention times for UGTs 1A1, 1A3, 1A4 and 2B10 were identical to those observed for HLM and showed similar sensitivity to treatment with β-glucuronidase and 3N HCl solution. As observed for CLZ in incubations with HLM, the UGT1A1-, 1A3-, 1A4- and UGT2B10-generated peaks were identified as CLZ-5'-glucuronide at 4.35 min and CLZ-N'-glucuronide at 5.2 min as determined by UPLC/MS/MS analysis (Figure 3-9).
One UGT exhibited detectable levels of activity against dmCLZ: the hepatic UGT 1A4. None of the other UGTs screened in our assays (UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15 or 2B17) exhibited any glucuronidation activity against dmCLZ using up to 1 mg of UGT-over-expressing cell homogenate. The UPLC retention time for UGT1A4 was identical to that observed for HLM and showed similar sensitivity to treatment with 3N HCl solution. Similar to that observed for dmCLZ observed in incubations with HLM, the UGT1A4-generated peak was identified as dmCLZ-5-N-glucuronide at 2.95 min as determined by UPLC analysis (Figure 3-10).
Figure 3-10 dmCLZ glucuronides formed by UGT-overexpressing cell lines

Glucuronidation assays were performed using 50 µg UGT1A1-overexpressing cell line homogenate, and 160 µM dmCLZ, and incubated at 37°C for 2 h with 4 mM UDP-glucuronic acid prior to analysis by UPLC as described in the Materials and Methods. **Panel A.** UGT1A4 + dmCLZ; **Panel B.** UGT1A4 + vehicle (1% DMSO). Peak 1, dmCLZ-5α-glucuronide; peak 2, dmCLZ; peak 3, UDP-glucuronic acid. AU: absorbance.

**UGT Kinetics**

Representative kinetic plots of CLZ glucuronidation rate versus substrate concentration are shown in Figure 3-11 for wild-type UGTs 1A1 and 1A4.
Figure 3-11 Kinetic curves for wild-type UGT1A1 A(TA)7TA- and UGT1A424Pro/48Leu-overexpressing cell homogenates against CLZ

UGT1A4 was the only UGT to catalyze formation of the CLZ-5-N-glucuronide as detected by UV spectra. Both UGTs 1A1 and 1A4 make the CLZ-N\(^{+}\)-glucuronide. UGT1A1 had a significantly lower \(K_M\) than UGT1A4 for the formation of the CLZ-N\(^{+}\)-glucuronide (\(p<0.0001\), Table 3-2).

| Table 3-2 Kinetic analysis of CLZ glucuronidation in UGT-over-expressing cell lines |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| UGT variant | CLZ-5-N-glucuronide | CLZ-N\(^{+}\)-glucuronide | CLZ-glucuronide | | |
| | \(V_{\text{max}}\) (pmol/min/ug protein) | \(K_M\) (\(\mu\)M) | \(V_{\text{max}}/K_M\) | \(V_{\text{max}}\) (pmol/min/ug protein) | \(K_M\) (\(\mu\)M) | \(V_{\text{max}}/K_M\) |
| UGT1A1 | - | - | -0.0001 | 19 ± 0.01 | 66 ± 2 | 19 ± 0.6 |
| UGT1A4 | 42 ± 3 | 173 ± 19 | 0.3 ± 0.03 | 6 ± 0.6 | 768 ± 62 | 8 ± 0.8 | 46 ± 3 | 189 ± 21 | 0.3 ± 0.03 |

Representative kinetic curves were performed as described in the Materials and Methods using CLZ concentrations of 10, 20, 40, 80, 160, 306, and 612 \(\mu\)M.
UGT1A3- and UGT2B10-generated glucuronides were not within the limits of the ultraviolet (UV) detection system, therefore kinetics were not performed for this enzyme.

UGT1A4-mediated formation of the dmCLZ-5-N-glucuronide is seen in Figure 3-12.

Figure 3-12 Kinetic curves for wild-type UGT1A4\(^{24Pro/48Leu}\)-overexpressing cell homogenates against CLZ

![UGT1A4 Kinetic Curve](image)

Representative kinetic curves were performed as described in the Materials and Methods using dmCLZ concentrations of 10, 20, 40, 80, 160, 320, and 640 μM for cell lines.

Compared to its activity against CLZ, UGT1A4 has a 2.3-fold larger \(K_M\) for the formation of the dmCLZ-5-N-glucuronide compared to the formation of CLZ-5-N-glucuronide (Table 3-3).

Discussion

**OLZ Glucuronidation**

Similar to that observed *in vivo*, three primary OLZ glucuronide products were observed in HLM: OLZ-4’-N-glucuronide and two isomers of OLZ-10-N-glucuronide (254, 398). Previous
studies had shown that UGT1A4 is active against OLZ (33). However, only selected human UGTs (1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15) over-expressed in baculovirus-infected insect cells were examined (33). Using UGT-over-expressing HEK293 cell homogenates, all of the known human UGT1A and UGT2B enzymes except UGTs 1A5 and 2B28 were screened for activity against OLZ in the present study. In addition to UGT1A4, UGT2B10 was found to have glucuronidation activity against OLZ. The 3.6- and 4-fold larger $K_m$ observed for UGT2B10 versus UGT1A4 for OLZ-10-N-glucuronide isomer 1 and OLZ-10-N-glucuronide isomer 2, respectively, suggests that UGT1A4 is more important in their formation than UGT2B10.

UGT2B10 exhibited a 1.8-fold lower binding affinity in formation of the OLZ-4’-N-glucuronide as compared to UGT1A4. While the $V_{\text{max}}$ for OLZ-4’-N-glucuronide formation was 2.7-fold lower than for the reaction catalyzed by UGT1A4, previous studies demonstrated that UGT2B10 exhibits a 1.5 – 4.8-fold higher level of expression than UGT1A4 in human liver (306, 430, 431), suggesting that both enzymes may play an important role in OLZ-10-N-glucuronide and OLZ-4’-N-glucuronide formation.

**CLZ and dmCLZ Glucuronidation**

Similar to that observed in human metabolic studies, two primary CLZ-glucuronide products were observed after CLZ incubation with HLM – CLZ-5-N-glucuronide and CLZ-$N^\gamma$-glucuronide (27, 28, 429). Previous studies had shown that UGTs 1A3 and 1A4 are active against CLZ (432-435). However, to our knowledge, these were the only human UGTs screened for activity in these studies (432-435). Using UGT-over-expressing HEK293 cell homogenates, all of the known human UGT1A and UGT2B enzymes except UGTs 1A5 and 2B28 were screened for activity against OLZ in the present study. In addition to UGT1A3 and 1A4, UGTs 1A1 and 2B10 also exhibited glucuronidation activity against CLZ in the present study. Kinetic studies suggested that UGT1A4 is more important in the formation of CLZ-5-N-glucuronide than the
other UGTs examined. However, for the CLZ-\(N^+\)-glucuronide, the \(K_M\) for UGT1A4 was 12-fold greater than UGT1A1, suggesting that UGT1A1 is the most active hepatic UGT in terms of CLZ-\(N^+\)-glucuronide formation. As previously reported, UGT1A4 was found to form a quaternary glucuronide of dmCLZ. Previously only UGTs 1A3 and 1A4 were screened for activity against dmCLZ (435). In this study, we showed that UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17 exhibited no detectable glucuronidation activity against dmCLZ in our experimental parameters. Interestingly, UGT1A4 was less efficient at glucuronidating dmCLZ than CLZ, which may have implications for clearance of this active metabolite compared to CLZ clearance \textit{in vivo}. Previous \textit{in vivo} studies have shown that dmCLZ plasma levels do not correlate with CLZ dose.

The \(K_M\) and \(V_{\text{max}}\) obtained in this study of UGT1A1 and 1A4 against CLZ and UGT1A4 against dmCLZ were slightly higher than reported previously(434). This difference is likely due to the use of optimum enzyme pH (8.5) rather than physiological pH (7.0) in these previous studies. Thus, the kinetics studies on CLZ and dmCLZ revealed information regarding their likely \textit{in vivo} activities of active UGTs against these substrates.

\textbf{Conclusions}

UGTs 1A4 and 2B10 are the primary enzymes responsible for OLZ glucuronidation. UGTs 1A1, 1A3, 1A4, and 2B10 all exhibit glucuronidation activity against CLZ. Based on kinetic studies, UGTs 1A1 and 1A4 seem to be primarily involved in CLZ glucuronidation with UGT1A4 primarily catalyzing the formation of the CLZ-5-\(N\)-glucuronide and UGT1A1 primarily forming the CLZ-\(N^+\)-glucuronide. Only one UGT exhibited detectable glucuronidation against dmCLZ under our experimental screening conditions: UGT1A4. These results agree with previously published results and reveal novel UGTs that glucuronidate the severe weight gain-
inducing SGAs OLZ, CLZ, and its active metabolite dmCLZ. These results can be used to elucidate the role of UGT polymorphisms in SGA metabolism and guided the studies in Chapter 4.
Chapter 4

FUNCTIONAL CHARACTERIZATION OF POLYMORPHISMS OF UDP-GLUCURONOSYLT RANSFERASE ENZYMES RESPONSIBLE FOR THE GLUCURONIDATION OF SEVERE WEIGHT GAIN-INDUCING SECOND-GENERATION ANTIPSYCHOTICS

The studies in this chapter elucidate the kinetics of known functional variants of the UGTs shown in Chapter 3 to exhibit enzymatic activity against the severe weight gain-inducing SGAs OLZ, CLZ, and its active metabolite dmCLZ. It is the hypothesis of this study that functional variants of UGTs with enzymatic activity against OLZ, CLZ, and dmCLZ will significantly alter the kinetic measures of enzyme maximum velocity, affinity, and efficiency compared to wild-type UGT enzymes.

Introduction

There is large interindividual variability in OLZ (20, 21, 34-37), CLZ, and dmCLZ clearance (17, 31). Studies indicate clinical outcomes and plasma concentrations are related (17, 21, 31). Weight gain and obesity occur only in a subset of patients taking CLZ or OLZ (38). Evidence suggests that there is a dose-response relationship between CLZ or OLZ serum concentrations and metabolic outcomes (39-42). Plasma concentrations of OLZ, CLZ, and dmCLZ do not correlate well with administered dose, indicating an important role of drug metabolizing enzymes in determining plasma levels and interindividual variation in plasma levels when subjects receive the same dose. Given the significant role of UGT enzymes in OLZ, CLZ, and dmCLZ metabolism as evidenced by drug metabolic product percentages, it is relevant to examine the effect of functional UGT polymorphisms. UGT1A4 is a hepatic enzyme that exhibits
prevalent functional missense polymorphisms (425, 436, 437). Specifically, the codon 24 proline>threonine variant (UGT1A4<sup>24Thr/48Leu</sup>) and the codon 48 leucine>valine variant (UGT1A4<sup>24Pro/48Val</sup>) have allelic frequencies of 8 and 9% in Caucasians respectively (437, 438). HEK293 cell lines over-expressing the UGT1A4<sup>24Thr/48Leu</sup> and UGT1A4<sup>24Pro/48Val</sup> variants were described previously (418, 419, 421, 424, 427, 437). Similar to that described for UGT1A4, a prevalent missense polymorphism (10% in Caucasians) also exists for UGT2B10 at codon 67 (aspartic acid>tyrosine, UGT2B10<sup>67Y</sup>) (421, 427, 430). A HEK293 cell line over-expressing the UGT2B10<sup>67Y</sup> variant has been described previously (421, 427). The goal of these studies was to examine the potential effects of prevalent SNPs in active UGTs on formation of individual OLZ, CLZ, and dmCLZ glucuronide metabolites. The results of this study demonstrate that prevalent missense SNPs in UGTs 1A4 and 2B10 may be important in the overall metabolism of OLZ, CLZ, and dmCLZ.

**Materials and Methods**

*Chemicals and Materials.*

All chemicals and materials were purchased as described in Chapter 3.

*Cell Lines*

The cell lines over-expressing the UGT1A and UGT2B isoforms used in this study were described previously (421-426) and were grown, prepared, and stored as described in Chapter 3.
**Western Blot Analysis**

UGT1A4 and UGT2B10 protein levels were determined by Western blot analysis for variant UGT1A4-overexpressing cell lines as described previously (425, 426) and are described briefly in Chapter 3.

**Glucuronidation Assays**

Assays were performed as described in Chapter 3. UGT1A4$^{24P48V}$ kinetic assays were performed using 12.5 μg of over-expressing cell homogenate protein for 30 min as these conditions were in the linear range of enzyme activity. UGT1A4$^{24T48L}$ and UGT2B10$^{67Y}$ assays were performed as described for their wild-type counterparts. As controls, glucuronidation assays were performed using HLM as a positive control for glucuronidation activity and untransfected HEK293 cell homogenate protein as a negative control for glucuronidation activity. Four independent experiments were performed for kinetic analysis of UGT-over-expressing cell homogenates, with all assays within each experiment performed in duplicate.

**Statistical Analysis**

Michaelis-Menten kinetic constants were determined using Prism Version 5 software (La Jolla, CA). The two-sample t-test (two-tailed) was used to compare kinetic values of glucuronide formation for the UGT1A4 and 2B10 isoforms against OLZ in cell lines.
Results

Analysis of OLZGglucuronidation by Hepatic UGT Variants

After normalization of UGT1A4 variant protein levels in UGT1A4-over-expressing cell homogenates by Western blot analysis, no significant difference in $V_{\text{max}}/K_M$ for OLZ-4'-N-glucuronide formation was observed between the UGT1A4$^{24\text{Pro/48Val}}$ variant and either of the other two UGT1A4 isoforms (Table 4-1). The $V_{\text{max}}/K_M$ of the UGT1A4$^{24\text{Pro/48Val}}$ variant was 3.6- ($p<0.0001$) and 11- ($p<0.0001$) fold higher than that observed for wild-type UGT1A4$^{24\text{Pro/48Leu}}$ and the UGT1A4$^{24\text{Thr/48Leu}}$ variant, respectively, for formation of OLZ-10-N-glucuronide isomer 1, and 4.7- ($p<0.0001$) and 4.3- ($p<0.0001$) fold higher, respectively, for OLZ-10-N-glucuronide isomer 2 formation (Table 4-1). While a significant ($p<0.0001$) 3-fold lower $V_{\text{max}}/K_M$ was observed for the UGT1A4$^{24\text{Thr/48Leu}}$ variant versus the wild-type UGT1A4$^{24\text{Pro/48Leu}}$ for formation of OLZ-10-N-glucuronide isomer 1, no difference was observed for OLZ-10-N-glucuronide isomer 2 or the OLZ-4'-N-glucuronide.

Table 4-1 Kinetic analysis of UGT1A4 and UGT2B10 variants against OLZ in vitro

<table>
<thead>
<tr>
<th>UGT variant</th>
<th>OLZ-10-N-glucuronide isomer 1</th>
<th>OLZ-10-N-glucuronide isomer 2</th>
<th>OLZ-4'-N-glucuronide</th>
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<td></td>
<td>$V_{\text{max}}$ (pmol/min/mg)</td>
<td>$K_M$ (μM)</td>
<td>$V_{\text{max}}/K_M$ (pmol/min/μM)</td>
</tr>
<tr>
<td>UGT1A4$^{24\text{Pro}}$</td>
<td>145 ± 12</td>
<td>156 ± 17</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>UGT1A4$^{24\text{Pro/48Val}}$</td>
<td>190 ± 24</td>
<td>584 ± 67</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>UGT1A4$^{24\text{Thr/48Leu}}$</td>
<td>223 ± 5</td>
<td>69 ± 6.5</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>UGT2B10$^{290\text{Thr}}$</td>
<td>0.35 ± 0.19</td>
<td>564 ± 15</td>
<td>0.0006 ± 0.001</td>
</tr>
<tr>
<td>UGT2B10$^{290\text{Leu}}$</td>
<td>no activity detected</td>
<td>no activity detected</td>
<td>no activity detected</td>
</tr>
</tbody>
</table>

$^a$ For UGT2B10 variants, value represents $K_M/V_{\text{max}}$ due to lack of availability of an UGT2B10 antibody to accurately quantify UGT2B10 protein levels

$^b$ $p=0.0001$, $^c$ $p=0.0018$ versus corresponding value for UGT1A4$^{24\text{Pro/48Val}}$, $^d$ $p=0.0001$ versus corresponding value for UGT1A4$^{24\text{Pro/48Leu}}$
Real-time PCR and Western analysis demonstrated similar levels of UGT2B10 expression in the UGT2B10 wild-type and codon 67 variant-over-expressing cell lines (Figure 3-5, Figure 4-1) in agreement with levels previously published for these cell lines (421, 422).

Figure 4-1 Real-time PCR relative quantification of HEK293 UGT-overexpressing cell lines

Unlike the activity observed for the wild-type UGT2B10^{67Asp}, no glucuronidation activity was observed for the UGT2B10^{67Tyr} variant against OLZ (Table 4-1).

Analysis of CLZ and dmCLZ Glucuronidation by Hepatic UGT Variants

After normalization of the UGT1A4 variant protein levels in UGT1A4-over-expressing cell homogenates by Western blot analysis, significant differences in $V_{max}/K_M$ for CLZ-glucuronide formation were observed between the UGT1A4^{24Pro/48Val} variant and both of the other two UGT1A4 isoforms (Table 4-2).
### Table 4-2 Kinetic analysis of UGT1A4 variants against CLZ in vitro

<table>
<thead>
<tr>
<th>UGT variant</th>
<th>CLZ-5-N-glucuronide</th>
<th>CLZ-N⁺-glucuronide</th>
<th>CLZ-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (pMol/min/µg)</td>
<td>$K_M$ (µM)</td>
<td>$V_{\text{max}}/K_M$</td>
</tr>
<tr>
<td>UGT1A4²⁴Pro/48Leu</td>
<td>42 ± 3</td>
<td>173 ± 19</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>UGT1A4²⁴Thr/48Leu</td>
<td>33 ± 3</td>
<td>167 ± 19</td>
<td>0.2 ± 0.01**</td>
</tr>
<tr>
<td>UGT1A4²⁴Pro/Val</td>
<td>117 ± 18</td>
<td>73 ± 11</td>
<td>1.7 ± 0.4**</td>
</tr>
</tbody>
</table>

* $p<0.04$, ** $p<0.001$, * $p<0.0003$, ** $p<0.0008$ versus corresponding value for UGT1A4²⁴Pro/48Leu

The $V_{\text{max}}/K_M$ of the UGT1A4²⁴Pro/48Val variant was 5- (p=0.0003, p=0.0003) fold higher than that observed for wild-type UGT1A4²⁴Pro/48Leu and the UGT1A4²⁴Thr/48Leu variant, respectively, for formation of CLZ-N⁺-glucuronide, 5.7- (p=0.001) and 8.5- (p=0.0008) fold higher, respectively, for CLZ-5-N-glucuronide formation, and 5.5- (p=0.0008) and 8.2- (p=0.0007) fold higher, respectively, for all CLZ-glucuronide formation (Table 4-2). While a significant (p=0.04) 1.5-fold lower $V_{\text{max}}/K_M$ was observed for the UGT1A4²⁴Thr/48Leu variant versus the wild-type UGT1A4²⁴Pro/48Leu for formation of CLZ-5-N-glucuronide and for all CLZ-glucuronide formation, no difference was observed for CLZ-N⁺-glucuronide.

Similarly, significant differences in $V_{\text{max}}/K_M$ for dmCLZ-5-N-glucuronide formation were observed between the UGT1A4²⁴Pro/48Val variant and both of the other two UGT1A4 isoforms (Table 4-3).
Table 4-3 Kinetic analysis of UGT1A4 variants against dmCLZ in vitro

<table>
<thead>
<tr>
<th>UGT variant</th>
<th>( V_{\text{max}} ) (pmol·min(^{-1})·µg(^{-1}))</th>
<th>( K_M ) (µM)</th>
<th>( \frac{V_{\text{max}}}{K_M} ) (nl·min(^{-1})·µg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A4(^{24\text{Pro/48Leu}})</td>
<td>10 ± 0.7</td>
<td>379 ± 46</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>UGT1A4(^{24\text{Thr/48Leu}})</td>
<td>6 ± 0.9</td>
<td>440 ± 45</td>
<td>15 ± 1*</td>
</tr>
<tr>
<td>UGT1A4(^{24\text{Pro/48Val}})</td>
<td>17 ± 1**</td>
<td>173 ± 22**</td>
<td>100 ± 8**</td>
</tr>
</tbody>
</table>

\( ^*p=0.0003, \ ^{**p<0.0001, \ ^{***p<0.0001} \text{versus corresponding value for UGT1A4}^{24\text{Pro/48Leu}} \text{variant} \)

The \( \frac{V_{\text{max}}}{K_M} \) of the UGT1A4\(^{24\text{Pro/48Val}}\) variant was 3.3- \( p<0.0001 \) and 6.7- \( p<0.0001 \) fold higher than that observed for wild-type UGT1A4\(^{24\text{Pro/48Leu}}\) and the UGT1A4\(^{24\text{Thr/48Leu}}\) variant, respectively, for formation of dmCLZ-5-N-glucuronide (Table 4-3). A significant \( (p=0.0003) \) 2-fold lower \( \frac{V_{\text{max}}}{K_M} \) was observed for the UGT1A4\(^{24\text{Thr/48Leu}}\) variant versus the wild-type UGT1A4\(^{24\text{Pro/48Leu}}\) for formation of dmCLZ-5-N-glucuronide (Table 4-3).

**Discussion**

*OLZ Glucuronidation by Hepatic UGT Variants*

The present study is the first to examine the functional importance of missense SNPs in relevant UGTs on OLZ *in vitro*. HEK293 cell homogenates over-expressing the UGT1A4\(^{24\text{Pro/48Val}}\) variant exhibited significantly higher levels of OLZ-10-N-glucuronide formation (both isomers 1 and 2). These data are consistent with results from a recent study demonstrating a lower level of plasma OLZ in subjects with the more active detoxifying UGT1A4\(^{48\text{Val}}\)-encoding *3 allele (36). A discernable effect of the UGT1A4\(^{24\text{Pro/48Val}}\) variant on OLZ-4'-N-glucuronide formation was not similarly observed *in vitro* in over-expressing cell homogenates. Cell homogenates over-expressing the UGT1A4\(^{24\text{Thr/48Leu}}\) variant exhibited a significant decrease in OLZ-10-N-
glucuronide isomer 1 formation compared with wild-type UGT1A4<sup>24Pro/48Leu</sup>. Together, these data suggest that the UGT1A4 Pro24Thr and Leu48Val polymorphisms are prevalent UGT1A4 missense SNPs that may be linked to altered hepatic OLZ glucuronidation activity.

Cell lines over-expressing the UGT2B10<sup>67Tyr</sup> variant exhibited no glucuronidation activity against OLZ. This is similar to that observed for this polymorphism against a number of UGT2B10 substrates including nicotine, cotinine and tobacco-specific nitrosamines and is consistent with this variant being a functional ‘knock-out’ of UGT2B10 enzyme activity (24, 421, 427, 430, 439, 440). This suggests that the UGT2B10<sup>67Tyr</sup> variant may be an important determinant of the ability of an individual to glucuronidate CLZ.

**CLZ and dmCLZ Glucuronidation by Hepatic UGT Variants**

The present study is the first to examine the functional importance of missense SNPs in relevant UGTs on CLZ and dmCLZ in vitro at physiological conditions. Similar to that observed for OLZ, HEK293 cell homogenates over-expressing the UGT1A4<sup>24Pro/48Val</sup> variant exhibited significantly higher levels of CLZ-5-N-glucuronide, CLZ-N<sup>+</sup>-glucuronide and dmCLZ-5-N-glucuronide formation. The kinetics of the UGT1A4<sup>48Val</sup> variant were previously studied in vitro for the CLZ-5-N-glucuronide and CLZ-N<sup>+</sup>-glucuronide combined (441). Our reported enzyme efficiency for overall CLZ-glucuronidation (CLZ-5-N-glucuronide and CLZ-N<sup>+</sup>-glucuronide combined) is in the same direction of change but greater than that reported previously. This may be due to the different experimental parameters used previously compared to this study. Cell homogenates over-expressing the UGT1A4<sup>24Thr/48Leu</sup> variant exhibited a significant decrease in OLZ-5-N-glucuronide compared with wild-type UGT1A4<sup>24Pro/48Leu</sup>. Together, these data suggest that the UGT1A4 Pro24Thr and Leu48Val polymorphisms are prevalent UGT1A4 missense SNPs that may be linked to altered hepatic CLZ glucuronidation activity.
Conclusions

We have identified three functional UGT variants that significantly alter the kinetics of UGT1A4 and 2B10 enzyme activity against OLZ, CLZ, and dmCLZ. These polymorphisms result in differential enzyme activity, which may result in altered glucuronidation activity in HLM \textit{in vitro} and \textit{in vivo}. 
Chapter 5

GENOTYPE: PHENOTYPE ANALYSIS OF HLM GLUCURONIDATION ACTIVITIES AGAINST SEVERE WEIGHT GAIN-INDUCING SECOND-GENERATION ANTIPSYCHOTICS

Introduction

The prevalence of the UGT1A1*28, UGT1A4*2 (UGT1A4<sup>24Thr/48Leu</sup>), UGT1A4*3 (UGT1A4<sup>24Pro/48Val</sup>), and UGT2B10*2 (UGT2B10<sup>67Tyr</sup>) variant alleles is ~30%, 8%, 9%, and 10% in Caucasians, respectively (421, 427, 436, 437, 442). To explore a possible in vivo relationship between OLZ, CLZ, and dmCLZ glucuronidation and these UGT1A1, UGT1A4 and UGT2B10 polymorphisms, a series of 113 HLM were examined for their glucuronidation activity against OLZ, CLZ, or dmCLZ.

Materials and Methods

Chemicals and Materials.

All chemicals and materials were purchased as described in Chapter 3. Lamotrigine (LTG) was purchased from Sigma-Aldrich (St. Louis, MO).

Tissues

The normal human liver tissue specimens used for these studies have been described previously (418, 419) and methods for collection, processing, storage of these tissues was the same as described in Chapter 3.
**UGT Genotyping**

Genotyping for UGT1A4 and UGT2B10 has been previously described for all of the HLM specimens described in this study (418, 421, 422). Briefly, UGT1A4 genotypes were determined by direct sequencing of PCR-amplified PCR products from liver genomic DNA spanning both codons 24 and 48 for UGT1A4. The same primers were used for both PCR amplification and sequencing of UGT1A4: sense, 5'-GGCTTCTGCTGAGATGGCCAG-3', and antisense, 5'-CCTTGAGTGTAGCCCAGCGT-3', corresponding to nucleotides located –13 to +8 and +277 to +306, respectively, relative to the UGT1A4 translation start site (Genbank accession no. NM_007120). Sequencing was performed using an ABI 3130 Capillary Sequencer at the Functional Genomics Core Facility at the Penn State College of Medicine. Restriction fragment length polymorphism (RFLP) analysis of the UGT2B10 codon 67 polymorphism (SNP 199G>T) was performed to identify individuals with the UGT2B10 codon 67 polymorphism as described previously (421). PCR amplification was performed on genomic DNA using a sense primer (5'-AAGGATGGCTCTGAAATGGACTA-3') and an antisense primer (5'-ATGAGTAGCCAGGACTGAAGCTGT-3') corresponding to nucleotides -4 to +19 and +535 to +512, respectively, relative to the UGT2B10 translation start site. The 539 bp PCR product was subjected to digestion with Hinfl (New England Biolabs) at 37°C for 3 h. An endogenous Hinfl restriction enzyme site present within this PCR-amplified fragment acted as a control for enzyme digestion. A migration pattern of 426 and 113 bp is indicative of the polymorphic SNP199T variant, whereas a migration pattern of 222, 204, and 113 bp is indicative of the wild-type SNP199G. The region spanning the UGT1A1 TATAA box promoter element was PCR-amplified (PCR product, 98–100 bp) as described previously (443) with sense (1A1S, 5'-GTCACGTGACACAGTCAAAC-3') and antisense (1A1AS, 5'-TTTGCTCCTGCCAGAGGTT-
3′) primers corresponding to nucleotides −104 through −85 and −25 through −7, respectively, relative to the translation start site in UGT1A1 exon 1 (GenBank accession no. AF297093). PCR amplifications were routinely performed in a 50-μl reaction volume containing 150 ng of purified genomic DNA, 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 2.5 mm MgCl2, 0.2 mm each of deoxynucleotide trisphosphate, 20 pmol of both sense and antisense UGT1A1 primers, 5 μCi [α-35S]-dATP, and 2.5 units of Taq DNA polymerase. Samples were subjected to electrophoresis at 50 V/cm for 4 h; gels were dried and subsequently exposed for autoradiography for 1 week. Bands of either 98 or 100 bp, representing the six or seven (TA) repeats of UGT1A1*1 or UGT1A1*28, respectively, were visualized, and their size were determined by comparison with a 5′-end 32P-labeled 10-bp DNA ladder. Representative samples exhibiting each of the UGT1A1 TATAA box genotype patterns observed by electrophoresis were examined by dideoxy sequencing, with PCR products purified after electrophoresis in 2% agarose using the QIAEX II gel extraction kit and sequenced at the DNA Sequencing Facility in the Department of Genetics at the University of Pennsylvania Medical Center using the same sense and antisense primers as were used for PCR amplification. Because the prevalences of non-UGT1A1*28 variant alleles are low in the population, all UGT1A1 genotyping for the Gilbert’s syndrome polymorphism that exhibited a pattern consistent with the wild-type A(TA)6TAA promoter region was considered to be UGT1A1*1.

**Glucuronidation Assays**

Homogenates or HLM were incubated with alamethicin (50 μg/mg protein) for 15 min on ice, similar to that described previously (425, 427). Glucuronidation and kinetic assays were performed as described in Chapter 3. To test the relative contribution of UGT1A4 in overall OLZ glucuronidation, five HLM exhibiting the UGT1A4(*1/*1)/UGT2B10 (*1/*1) genotype were
randomly selected and co-treated with 300 µM OLZ and 2000 µM of the UGT1A4 inhibitor LTG, a concentration within the linear range of kinetic analysis for HLM against OLZ. Two independent experiments were performed for rate determination assays and three for kinetic analysis for HLM specimens.

Statistical Analysis

Michaelis-Menten kinetic constants were determined using Prism Version 5 software (La Jolla, CA). The two-sample t-test (two-tailed) was used to compare kinetic values of glucuronide formation for the UGT isoforms against OLZ, CLZ and dmCLZ in cell lines and HLMs. To perform the most conservative comparison, unequal variances were assumed when comparing levels of OLZ- , CLZ- , or dmCLZ-glucuronide formation in HLMs with wild-type alleles versus HLM with one or two polymorphic alleles. All the levels of OLZ-glucuronide formation were power transformed (using a power of 1/1.5) to make sure the data was approximately normally distributed. Analysis of OLZ glucuronidation in HLM stratified by UGT1A4 genotypes was performed only for those specimens also exhibiting the wild-type UGT2B10 (*1/*1) genotype (n=92); similarly, analysis of OLZ glucuronidation in HLM stratified by UGT2B10 genotypes was performed only for those specimens also exhibiting the wild-type UGT1A4 (*1/*1) genotype (n=95). Regression analysis of OLZ-glucuronide formation against genotype was performed (SAS Corporation, Cary, NC). Analysis CLZ-N+ -glucuronide formation in HLM stratified by UGT1A4 genotypes was performed only for those specimens also exhibiting the wild-type UGT1A1 (*1/*1) genotype (n=40); similarly, analysis of CLZ glucuronidation in HLM stratified by UGT1A1 genotypes was performed only for those specimens also exhibiting the wild-type UGT1A4 (*1/*1) genotype (n=96).
Results

Analysis of OLZ Glucuronidation by HLM Stratified by UGT1A4 or UGT2B10 Genotypes

Comparing HLM homozygous for the wild-type UGT2B10<sup>67Asp</sup> allele [UGT2B10 (*1/*1) genotype (n=92)], no significant difference in formation of any OLZ glucuronide was observed when comparing those homozygous for the UGT1A4<sup>24Thr/48Leu</sup> allele [UGT1A4 (*2/*2) genotype; n=2] to HLM homozygous for the UGT1A4<sup>24Pro/48Leu</sup> allele [UGT1A4 (*1/*1) genotype; n=73], even after excluding HLM with one or more UGT1A4<sup>24Pro/48Val</sup> alleles (results not shown). Therefore, for further analysis of the UGT1A4*3 and UGT2B10<sup>67Tyr</sup> alleles, HLM were not sub-stratified based on the UGT1A4*2 allele.

Of the 113 HLM, 105 were included in this study as they were heterozygous or homozygous for only one of the variant genotypes of interest, allowing us to study the effects of each genetic variant individually. For HLM homozygous for the wild-type UGT2B10<sup>67Asp</sup> (*1/*1) genotype, there was a significant 2.1- (p=0.04) and 1.6- (p=0.0017) fold increase in formation of the OLZ-10-N-glucuronide isomer 1 and the OLZ-4’-N-glucuronide, respectively (Figure 5-1, panels A and C) and a significant (p=0.02) 2.0-fold increase in overall OLZ glucuronidation activity (Figure 5-1, panel D) in HLM with the UGT1A4 (*3/*3) genotype (n=2) as compared with HLM with the UGT1A4 (*1/*1) genotype (n=82).
Figure 5-1 HLM activity stratified by UGT genotypes

Shown are the levels of OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, and OLZ-4'-N-glucuronide formation versus UGT1A4 or UGT2B10 genotypes in HLM. Glucuronidation activity assays were performed using 300 μM OLZ and 12.5 μg of HLM protein, and OLZ glucuronides were detected and separated by UPLC as described in the Materials and Methods. Using genomic DNA from the same liver specimens for which HLMs were prepared, UGT1A4 and UGT2B10 genotypes were determined using DNA sequencing and RFLP analysis, respectively. Panels A-D, OLZ glucuronidation in HLM stratified by UGT1A4 genotypes; panels E-H, OLZ glucuronidation in HLM stratified by UGT2B10 genotypes. Panel A and E, OLZ-10-N-glucuronide isomer 1 formation; panels B and F, OLZ-10-N-glucuronide isomer 2 formation; panels C and G, OLZ-4'-N-glucuronide formation; panels D and H, all OLZ-glucuronide product formation. Analysis of OLZ glucuronidation in HLM stratified by UGT1A4 genotypes was performed only for those specimens also exhibiting the wild-type UGT2B10 (1*1) genotype (n=92); similarly, analysis of OLZ glucuronidation in HLM stratified by UGT2B10 genotypes was performed only for those specimens also exhibiting the wild-type UGT1A4 (1*1) genotype (n=95).
While a 2.2-fold increase in OLZ-10-N-glucuronide isomer 2 formation was observed for HLM with the UGT1A4 (*3/*3) genotype versus HLM with the UGT1A4 (*1/*1) genotype, this difference was not significant ($p=0.09$). Regression analysis showed that the UGT1A4 *3 allele is a significant predictor for formation of the OLZ-10-N-glucuronide isomer 1 ($p=0.0047$), the OLZ-10-N-glucuronide isomer 2 ($p=0.0001$), the OLZ-4′-N-glucuronide ($p=0.02$), and overall OLZ glucuronidation ($p=0.0013$) (Table 5-1).

**Table 5-1 Regression analysis of OLZ-glucuronidation (power transformed) against the UGT1A4 *3 allele**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter Estimate</th>
<th>T-Statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLZ-10-N-glucuronide isomer 1</td>
<td>2.0854</td>
<td>2.8966</td>
<td>0.0047</td>
</tr>
<tr>
<td>OLZ-10-N-glucuronide isomer 2</td>
<td>1.7021</td>
<td>3.9744</td>
<td>0.0001</td>
</tr>
<tr>
<td>OLZ-4′-N-glucuronide</td>
<td>1.1431</td>
<td>2.3670</td>
<td>0.0201</td>
</tr>
<tr>
<td>All OLZ-glucuronide products</td>
<td>3.3967</td>
<td>3.3086</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

For each UGT1A4 *3 allele, product formation increased by 3.0, 3.3, 1.2, and 7.9 pmol min$^{-1}$ mg protein$^{-1}$ for OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, OLZ-4′-N-glucuronide, and overall glucuronidation, respectively, representing a 16, 25, 10, and 15% increase in glucuronidation for each UGT1A4 *3 allele compared to the mean glucuronide-product formation by wild-type samples, respectively.

For HLM with the wild-type UGT1A4 (*1/*1) genotype (n=95), there was only a single HLM with the UGT2B10 (*2/*2) genotype. Therefore, HLM with the UGT2B210 (*1/*2) (n=12) or (*2/*2) (n=1) genotypes were combined into one group for analysis of OLZ glucuronidation activity stratified by UGT2B10 genotype. There was a significant 1.9-fold decrease in both OLZ-10-N-glucuronide isomer 1 ($p=0.0016$; Figure 5-1, panel E) and isomer 2 ($p=0.0013$; Figure 5-1, panel F) formation, a significant 2.7-fold decrease in OLZ-4′-N-glucuronide formation ($p<0.0001$; Figure 5-1 panel G), and a significant 2.1-fold decrease in overall OLZ...
glucuronidation ($p=0.0002$; Figure 5-1, panel H) in UGT1A4 (*1/*1) HLMs with at least one UGT2B10*2 allele as compared to UGT1A4 (*1/*1) HLMs with the UGT2B10 (*1/*1) genotype. The single HLM with a combined UGT1A4 (*1/*1)/UGT2B10 (*2/*2) genotype exhibited a 2.6-, 2.1-, and 4.1-fold decrease in the formation of OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, and OLZ-4’-N-glucuronide, respectively, as compared to UGT1A4 (*1/*1) HLMs with the UGT2B10 (*1/*1) genotype, suggesting a trend towards decreasing OLZ glucuronidation activity with increasing numbers of the UGT2B10*2 allele. Regression analysis showed that the UGT2B10*2 allele was a significant predictor for formation of the OLZ-10-N-glucuronide isomer 1 ($p=0.002$), the OLZ-10-N-glucuronide isomer 2 ($p=0.0019$), the OLZ-4’-N-glucuronide ($p<0.0001$), and overall OLZ glucuronidation ($p<0.0001$) (Table 5-2).

Table 5-2  Regression analysis of OLZ-glucuronidation (power transformed) against the UGT2B10 *2 allele

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter Estimate</th>
<th>T-Statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLZ-10-N-glucuronide isomer 1</td>
<td>-2.2686</td>
<td>-3.1110</td>
<td>0.0025</td>
</tr>
<tr>
<td>OLZ-10-N-glucuronide isomer 2</td>
<td>-1.3568</td>
<td>-3.0824</td>
<td>0.0027</td>
</tr>
<tr>
<td>OLZ-4’-N-glucuronide</td>
<td>-2.3452</td>
<td>-4.7905</td>
<td>0.0000</td>
</tr>
<tr>
<td>All OLZ-glucuronide products</td>
<td>-4.2194</td>
<td>-4.0023</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

For each UGT2B10 *2 allele, product formation decreased by 4.1, 2.0, 4.3, and 10.5 pmol min⁻¹ mg protein⁻¹ for OLZ-10-N-glucuronide isomer 1, OLZ-10-N-glucuronide isomer 2, OLZ-4’-N-glucuronide, and overall glucuronidation, respectively, representing a 20, 20, 50, and 30% decrease in glucuronide formation for each UGT2B10 *2 allele compared to the mean glucuronide-product formed by wild-type samples, respectively. There was insufficient power to examine the effect of combined UGT1A4 and UGT2B10 genotypes on OLZ glucuronidation in this series of HLM specimens.
To better assess the relative contribution of UGT1A4 to OLZ glucuronidation, five HLM were co-incubated with 300 µM OLZ and 2000 µM of the UGT1A4-specific inhibitor, LTG (33, 444). Overall OLZ glucuronidation was inhibited by 12% (p=0.009) in UGT1A4 (*1/*1)/UGT2B10 (*1/*1) HLM incubated with LTG (Figure 5-2).

Using assay conditions (300 µM OLZ and 2000 µM LTG) similar to those of previous studies (33), overall OLZ glucuronidation was inhibited by 41% (p=0.02) in homogenates from UGT1A4-over-expressing cells and by an average of 12% (p=0.009, n=3) in HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*1) genotype (Figure 5-2).

Results of kinetic analysis of HLM with varying UGT1A4 and UGT2B10 genotypes are shown in Table 5-3.
Table 5-3Kinetics analysis of OLZ glucuronidation by HLM with varying UGT1A4 and UGT2B10 genotypes

<table>
<thead>
<tr>
<th>HLM genotype</th>
<th>OLZ-10-N-glucuronide isomer 1</th>
<th>OLZ-10-N-glucuronide isomer 2</th>
<th>OLZ-4'-N-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{\text{max}}) (mol/min/mg)</td>
<td>(K_{\text{M}}) ((\mu)M)</td>
<td>(V_{\text{max}}/K_{\text{M}})</td>
</tr>
<tr>
<td>UGT1A4(*1/*1)/UGT2B10(*1/*1)</td>
<td>94.0 ± 14</td>
<td>352 ± 59</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td>UGT1A4(*1/*3)/UGT2B10(*1/*1)</td>
<td>160 ± 14</td>
<td>328 ± 33</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>UGT1A4(*3/*3)/UGT2B10(*1/*1)</td>
<td>188 ± 23</td>
<td>268 ± 41</td>
<td>0.70 ± 0.07</td>
</tr>
<tr>
<td>UGT2B10(*1/*2)/UGT1A4(*1/*1)</td>
<td>56 ± 27</td>
<td>302 ± 50</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>UGT2B10(*2/*2)/UGT1A4(*1/*1)</td>
<td>69 ± 27</td>
<td>273 ± 20</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Three HLM with the UGT1A4 (*1/*1) genotype, three HLM with the UGT1A4 (*1/*3) genotype, and two HLM with the UGT1A4 (*3/*3) genotype were examined in this analysis. Only HLM with the UGT1B10 (*1/*1) genotype was used to compare varying UGT1A4 genotypes. HLMs of each genotype were chosen at random.

For HLM stratified by UGT1A4 genotypes, there was a significant 1.8- \((p=0.03)\) and 2.5- \((p=0.009)\) fold increased \(V_{\text{max}}/K_{\text{M}}\) for formation of the OLZ-10-N-glucuronide isomer 1 in UGT2B10 (*1/*1) HLM with the UGT1A4 (*1/*3) and UGT1A4 (*3/*3) genotypes, respectively, as compared to UGT2B10 (*1/*1) HLM with the UGT1A4 (*1/*1) genotype. This was manifested primarily via an increase in \(V_{\text{max}}\), with HLM with the UGT1A4 (*1/*3)/UGT2B10 (*1/*1) and UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotypes exhibiting a significant 1.7- \((p=0.0046)\) and 2.0- \((p=0.04)\) fold higher \(V_{\text{max}}\), respectively, for OLZ-10-N-glucuronide isomer 1. There was a significant trend of increasing activity as measured by \(V_{\text{max}}/K_{\text{M}}\) with increasing numbers of the UGT1A4*3 allele for formation of the OLZ-10-N-glucuronide isomer 1 \((p<0.0001)\). For formation of the OLZ-10-N-glucuronide isomer 2, there was a significant \((p=0.008)\) trend of increasing \(V_{\text{max}}/K_{\text{M}}\) with increasing numbers of the UGT1A4*3 allele \((p<0.0001)\). For the OLZ-4’-N-glucuronide, HLM with the UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotype exhibited a 1.8-fold higher \(V_{\text{max}}/K_{\text{M}}\) \((p=0.0078)\), manifested primarily via a 1.9-fold higher \(V_{\text{max}}\) \((p=0.028)\), as compared to HLM with the UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotype.
(*1/*1)/UGT2B10 (*1/*1) genotype (Table 5-3). A significant (p=0.0075) trend of increasing V_{max}/K_{M} for HLM with increasing numbers of the UGT1A4*3 allele was also observed.

The effect of UGT2B10 genotype on HLM glucuronidation activities was strongest for the OLZ-4′-N-glucuronide. There was a significant 3.3-fold lower V_{max}/K_{M} (p=0.02) for formation of the OLZ-4′-N-glucuronide in HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*2) genotype as compared to HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*1) genotype, an effect that was manifested by primarily by a significant decrease in V_{max} (p=0.01; Table 5-3). A significant (p=0.0004) 5.8-fold lower V_{max}/K_{M} was observed for HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*2) genotype when compared to HLM with the UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotype. While statistical analysis could not be performed for comparisons with the single HLM with the UGT1A4 (*1/*1)/UGT2B10 (*2/*2) genotype, this HLM exhibited a 1.7-, 5.5- and 9.8-fold lower V_{max}/K_{M} for formation of the OLZ-4′-N-glucuronide as compared to HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*2), UGT1A4 (*1/*1)/UGT2B10 (*1/*1) and UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotypes, respectively.

While significant alterations in OLZ-10-N-glucuronide isomer 1 kinetics were not observed when comparing UGT1A4 (*1/*1)/UGT2B10 (*1/*2) versus UGT1A4 (*1/*1)/UGT2B10 (*1/*1) HLM, a significant (p=0.005) 3.7-fold decrease in V_{max}/K_{M} was observed when comparing UGT1A4 (*1/*1)/UGT2B10 (*1/*2) versus UGT1A4 (*3/*3)/UGT2B10 (*1/*1) HLM. A similar pattern was observed for OLZ-10-N-glucuronide isomer 2, with HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*2) genotype exhibiting a near-significant (p=0.057) 2.6-fold lower V_{max}/K_{M} as compared to HLM with the UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotype. In both cases these differences were primarily due to significant (p<0.02) decreases in V_{max}. 
Analysis of CLZ and dmCLZ Glucuronidation by HLM Stratified by UGT Genotype

The prevalence of the UGT1A4*24Thr/48Leu, UGT1A4*24Pro/48Val and UGT1A1 A(TA)7TAA variants is ~8%, 9%, and 30% in Caucasians, respectively (421, 427, 436, 437). To explore a possible in vivo relationship between CLZ and dmCLZ glucuronidation and the UGT1A1 and UGT1A4 polymorphisms, a series of 113 HLM were examined for their glucuronidation activity against CLZ and dmCLZ. The rate of glucuronide formation was determined by UPLC using 160 μM CLZ and 300 μM dmCLZ, a concentration within the linear range of kinetic analysis for HLM. Comparing HLM homozygous for the wild-type UGT1A1 A(TA)6TAA allele [UGT1A1 (*1/*1) genotype (n=40)], no significant difference in formation of any CLZ or dmCLZ glucuronide was observed when comparing those homozygous for the UGT1A4*24Thr/48Leu allele [UGT1A4 (*2/*2) genotype; n=2] to HLM homozygous for the UGT1A4*24Pro/48Val allele [UGT1A4 (*1/*1) genotype; n=33], even after excluding HLM with one or more UGT1A4*3 (UGT1A4*24Pro/48Val) alleles (Figure 5-3, Figure 5-4).

Figure 5-3 HLM activity against CLZ stratified by the UGT1A4 *2 allele
Figure 5-4 HLM activity against dmCLZ stratified by the UGT1A4 *2 allele

Therefore, for further analysis of the UGT1A1*28 and UGT2B10*3 alleles, HLM were not sub-stratified based on the UGT1A4*2 allele.

For HLM homozygous for the wild-type UGT1A4*24*24Pro/48Leu (*1/*1) genotype, there was a significant 1.5- (p<0.05) fold decrease in formation of the CLZ-$N^\prime$-glucuronide (Figure 5-5, panel B) in HLM with the UGT1A1 (*28/*28) genotype (n=11) as compared with HLM with the UGT1A1 (*1/*1) genotype (n=37).
Figure 5-5 HLM activity against CLZ stratified by the UGT1A1 and UGT1A4 genotypes

There was a significant trend ($p=0.02$) of decreasing CLZ-$N^+$-glucuronide product formation with increasing numbers of the *28 allele. While a 1.4-fold decrease in CLZ-$5-N$-glucuronide formation was observed for HLM with the UGT1A4 (*28/*28) genotype versus HLM with the UGT1A1 (*1/*1) genotype, this difference was not significant ($p=0.1$) nor was the trend of decreasing CLZ-$5-N^+$-glucuronide product formation with increasing numbers of the *28 allele ($p=0.09$, panel A).

The UGT1A1 enzyme exhibited very low levels of activity against CLZ in forming the CLZ-$5-N$-glucuronide that were only detectable by MRM analysis. Thus, only the analysis
examining the effect of the UGT1A4*3 allele on the CLZ-N\textsuperscript{+}-glucuronide formation was substratified for the UGT1A1*28 allele. There was a significant 2.2-fold increase in CLZ-5-glucuronide (p<0.005; Figure 5-5, panel D) formation, a significant 1.7-fold increase in glucuronide formation (p<0.05; panel E) and a significant 2.2-fold increase in all CLZ-glucuronidation (p<0.005; panel F) in UGT1A4 (*3/*3) HLMs as compared to UGT1A4 HLMs. There was a significant trend of increasing CLZ-5-N-glucuronide (p=0.0002), CLZ-glucuronide (p=0.02), and overall CLZ-glucuronide (p=0.0003) product formation with increasing numbers of the UT1A4*3 allele. There was a significant 1.3-fold increase in 5-N-glucuronide formation (p<0.05);

Figure 5-6) in UGT1A4 (*1/*3) HLMs as compared to UGT1A4 (*1/*1) HLMs and a significant 2.3-fold increase in dmCLZ-5-N-glucuronide formation (p<0.005);

Figure 5-6) in UGT1A4 (*3/*3) HLMs as compared to UGT1A4 (*1/*1) HLMs.

Figure 5-6 HLM activity against dmCLZ stratified by UGT1A4 genotype
The trend for dmCLZ-5-N-glucuronide formation with increasing numbers of the UGT1A4*3 allele was significant \( p=0.0008 \).

Results of kinetic analysis of HLM with varying UGT1A1 and UGT1A4 genotypes are shown in Table 5-4.

Table 5-4 Kinetic analysis of CLZ glucuronidation by HLMs of varying UGT1A1 and UGT1A4 genotypes

<table>
<thead>
<tr>
<th>HLM genotype</th>
<th>CLZ-5-N-glucuronide</th>
<th></th>
<th>CLZ-( N^)-glucuronide</th>
<th></th>
<th>CLZ-glucuronide formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\max} )</td>
<td></td>
<td>( K_m )</td>
<td></td>
<td>( V_{\max}/K_m )</td>
</tr>
<tr>
<td></td>
<td>(pmol.min(^{-1}).mg(^{-1}))</td>
<td></td>
<td>(( \mu )M)</td>
<td></td>
<td>(pmol.min(^{-1}).mg(^{-1}))</td>
</tr>
<tr>
<td>UGT1A1(( <em>1</em>/1)) / UGT1A4(( <em>1</em>/1))</td>
<td>758 ± 22</td>
<td></td>
<td>238 ± 5</td>
<td></td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>UGT1A1(( <em>1</em>/2)) / UGT1A4(( <em>1</em>/1))</td>
<td>686 ± 43(^{\ddagger})</td>
<td></td>
<td>217 ± 44</td>
<td></td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>UGT1A1(( <em>2</em>/2)) / UGT1A4(( <em>1</em>/1))</td>
<td>684 ± 61(^{\ddagger})</td>
<td></td>
<td>234 ± 26(^{\ddagger})</td>
<td></td>
<td>2.9 ± 0.1(^{\ddagger})</td>
</tr>
<tr>
<td>UGT1A4(( <em>1</em>/3)) / UGT1A1(( <em>1</em>/1))</td>
<td>1088 ± 93(^{\ddagger})</td>
<td></td>
<td>187 ± 22</td>
<td></td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>UGT1A4(( <em>3</em>/3)) / UGT1A1(( <em>1</em>/1))</td>
<td>1573 ± 202(^{\ddagger})</td>
<td></td>
<td>212 ± 22(^{\ddagger})</td>
<td></td>
<td>13 ± 0.7</td>
</tr>
</tbody>
</table>

* Three HLM with the UGT1A1(\( *1*/1\)), the UGT1A1(\( *1*/2\)), and the UGT1A4(\( *3*/3\)) genotype were examined in this analysis. Only HLM with the UGT1A4(\( *1*/1\)) genotype was used to compare varying UGT1A4 genotypes. HLMs of each genotype were chosen at random.

** Three HLM with the UGT1A1(\( *1*/1\)) genotype, three HLM with the UGT1A4(\( *1*/3\)) genotype, and two HLM with the UGT1A4(\( *3*/3\)) genotype were examined in this analysis. Only HLM with the UGT1A4(\( *1*/1\)) genotype was used to compare varying UGT1A4 genotypes. HLMs of each genotype were chosen at random.

\(^{\ddagger}\) \( p<0.05 \); \(^{\ddagger\ddagger}\) \( p<0.005 \); \(^{\ddagger\ddagger\ddagger}\) \( p<0.0005 \); \(^{\star\star\star}\) \( p<0.00005 \) versus the corresponding value for HLM with the UGT1A1(\( *1*/1\)) / UGT1A4(\( *1*/1\)) genotype.
For HLM stratified by UGT1A1 genotypes, there were no significant differences in formation of the CLZ-5-N-glucuronide in UGT1A4 (*1/*1) HLM with the UGT1A1 (*28/*28) genotypes as compared to UGT1A4 (*1/*1) HLM with the UGT1A1 (*1/*1) genotype, although the test for trend of decreasing V_\text{max} approached significance (p=0.054). There was a significant 1.4- (p<0.005) fold decrease in V_\text{max}/K_M for formation of the CLZ-N'-glucuronide in UGT1A4 (*1/*1) HLM with the UGT1A1 (*28/*28) genotypes as compared to UGT1A4 (*1/*1) HLM with the UGT1A1 (*1/*1) genotype. This was manifested primarily via a decrease in V_\text{max}, with HLM with the UGT1A1 (*1/*28)/UGT1A4 (*1/*1) and UGT1A1 (*28/*28)/UGT1A4 (*1/*1) genotypes exhibiting a significant 1.4- (p<0.05) and 1.7- (p<0.01) fold lower V_\text{max}, respectively, for CLZ-N'-glucuronide. There was a significant trend of decreasing activity as measured by V_\text{max}/K_M and of V_\text{max} with increasing numbers of the UGT1A1*28 allele for formation of the CLZ-N'-glucuronide (p=0.005 and 0.002, respectively). For overall CLZ-glucuronide formation, there was a significant 1.1- (p<0.05) fold decrease in V_\text{max}/K_M for UGT1A4 (*1/*1) HLM with the UGT1A1 (*28/*28) genotypes as compared to UGT1A4 (*1/*1) HLM with the UGT1A1 (*1/*1) genotype and a significant 1.1- (p<0.05) and 1.2- (p<0.05) fold decrease in V_\text{max} in HLM with the UGT1A1 (*1/*28)/UGT1A4 (*1/*1) and UGT1A1 (*28/*28)/UGT1A4 (*1/*1) genotypes, respectively, compared to HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype. There was a significant (p=0.02) trend of decreasing V_\text{max} with increasing numbers of the UGT1A1*28 allele.

The UGT1A4 genotype affected on HLM glucuronidation activities for both CLZ-glucuronide products and all CLZ glucuronidation. There was a significant 1.8- and 4.1-fold higher V_\text{max}/K_M (p<0.005 for both) for formation of the CLZ-5-N-glucuronide in HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*3) and UGT1A1 (*1/*1)/UGT1A4 (*3/*3) genotypes, respectively, as compared to HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype, an
effect that was manifested primarily by a significant 1.4- and 2.1-fold increase in $V_{\text{max}}$ ($p<0.005$; Table 5-4), but also by a 1.3- ($p<0.05$) and 2.0- ($p<0.005$) fold decrease in $K_M$. There was a significant trend in increasing $V_{\text{max}}$ ($p=0.0004$), decreasing $K_M$ ($p=0.0007$), and increase in $V_{\text{max}}/K_M$ ($p<0.0001$) for formation of the CLZ-5-$N$-glucuronide with increasing numbers of the UGT1A4*3 allele. HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*3) and the UGT1A1 (*1/*1)/UGT1A4 (*3/*3) genotype exhibited a 1.8- and 2.7-fold higher $V_{\text{max}}/K_M$ ($p<0.05$ and $<0.005$, respectively) for formation of the CLZ-$N^+$-glucuronide compared to HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype. This was due to a 1.4- and 1.6-fold ($p<0.05$ for both) increase in $V_{\text{max}}$ and a 1.3- and 1.8-fold decrease ($p<0.05$ for both) in $K_M$. There was a significant trend of increasing $V_{\text{max}}$ ($p=0.01$), decreasing $K_M$ ($p=0.003$), and increasing $V_{\text{max}}/K_M$ ($p=0.0003$) with increasing numbers of the UGT1A4*3 allele. UGT1A1 (*1/*1)/UGT1A4 (*1/*3) and the UGT1A1 (*1/*1)/UGT1A4 (*3/*3) genotype HLM exhibited a 2.8- and 4.0- ($p<0.01$ and $<0.005$, respectively) fold increase in $V_{\text{max}}/K_M$ for all CLZ-glucuronides, an effect mediated by both a 1.5- and 2.0- ($p<0.05$ and $<0.005$, respectively) fold increase in $V_{\text{max}}$ and a 1.3- and 2.0- ($p<0.05$ and $<0.005$, respectively) fold decrease in $K_M$. There was a significant trend of increasing $V_{\text{max}}$ ($p=0.003$), decreasing $K_M$ ($p=0.0008$), and increasing $V_{\text{max}}/K_M$ ($p<0.0001$) with increasing numbers of the UGT1A4*3 allele as compared to HLM with the UGT1A1(*1/*1)/UGT1A4(*1/*1) genotype.

Compared to HLM with the UGT1A1(*1/*1)/UGT1A4(*3/*3) genotype, HLM with the UGT1A1(*28/*28)/UGT1A4(*1/*1) genotype exhibited a 4.5-, 3.7-, and 4.2- ($p<0.001$ for all) fold decrease in $V_{\text{max}}/K_M$ for the formation of the CLZ-5-$N$-glucuronide, CLZ-$N^+$-glucuronide, and all CLZ-glucuronides, respectively. These differences were mediated primarily by significant ($p<0.005$, $p<0.01$, and $p<0.005$ respectively) differences in $V_{\text{max}}$, which were 2.3-, 2.4- and 2.3-fold lower for HLM with the UGT1A4 (*28/*28)/UGT1A4 (*1/*1) genotype as compared to HLM with the UGT1A4 (*1/*1)/UGT1A4 (*3/*3) genotype.
HLM kinetic analysis for dmCLZ stratified by the UGT1A4*3 allele is shown in Table 5-5.
Table 5-5 Kinetic analysis of dmCLZ glucuronidation in HLMs of varying UGT1A4 genotypes

<table>
<thead>
<tr>
<th>HLM genotype</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$K_M$ (µM)</th>
<th>$V_{\text{max}}/K_M$ (µl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A4(*1/*1)</td>
<td>108 ± 6</td>
<td>357 ± 21</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>UGT1A4(*1/*3)</td>
<td>120 ± 11</td>
<td>214 ± 23</td>
<td>0.6 ± 0.01</td>
</tr>
<tr>
<td>UGT1A4(*3/*3)</td>
<td>175 ± 13</td>
<td>128 ± 4</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

*Three HLM with the UGT1A4 (*1/*1) genotype, three HLM with the UGT1A4 (*1/*3) genotype.
**Two HLM with the UGT1A4 (*3/*3) genotype were examined in this analysis.
HLMs of each genotype were chosen at random.

$p<0.005$, $^b$p<0.05 versus the corresponding value for HLM with the UGT1A4 (*1/*1) genotype.

For dmCLZ-5-N-glucuronide formation, HLM with the UGT1A4(*1/*3) and the UGT(*3/*3) genotypes exhibited a 2.0- and 4.7- (p<0.05 and <0.005, respectively) increase in $V_{\text{max}}/K_M$ as compared to HLM with the UGT1A4(*1/*1) genotype. This was mediated by a a 1.7-fold ($p<0.005$) decrease in $K_M$ for HLM with the UGT1A4(*1/*3) genotype and a 1.6-fold ($p<0.005$) increase in $V_{\text{max}}$ and a 2.9-fold ($p<0.005$) decrease in $K_M$ for HLM with the UGT1A4(*3/*3) genotype. There was a significant trend of increasing $V_{\text{max}}$ ($p=0.0007$), decreasing $K_M$ ($p<0.0001$), and increasing $V_{\text{max}}/K_M$ ($p<0.0001$) with increasing numbers of the UGT1A4*3 allele as compared to HLM with the UGT1A4(*1/*1) genotype.
Discussion

Analysis of OLZ Glucuronidation by HLM Stratified by UGT1A4 or UGT2B10 Genotypes

The $K_m$ for both isomers of OLZ-10-N-glucuronide of HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*1) genotype was inbetween that observed in cell lines over-expressing UGT1A4 and UGT2B10 in vitro, suggesting a combined effect of both UGT1A4 and UGT2B10 on OLZ-10-N-glucuronide formation. For the OLZ-4’-N-glucuronide, the $K_m$ for HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*1) genotype was similar to that observed in vitro for the UGT2B10-over-expressing cell line. In addition, UGT1A4 (*1/*1) HLM with one ‘knock-out’ UGT2B10*2 allele exhibited a 2.7-fold decrease in rate of OLZ-4’-N-glucuronide formation and a >3.2-fold decrease in $V_{max}/K_m$ as compared to UGT1A4 (*1/*1)/UGT2B10 (*1/*1) HLM. This decrease was even greater for the single UGT1A4 (*1/*1) HLM specimen exhibiting the UGT2B10 (*2/*2) genotype, with UGT1A4-dependent activity accounting for <50% of OLZ-10-N-glucuronide isomers and <20% of OLZ-4’-N-glucuronide formation when comparing the kinetics of this HLM specimen versus that observed for HLM with the UGT1A4 (*1/*1)/UGT2B10 (*1/*1) genotype.

UGT2B10 exhibited a ~2-fold decrease in $K_m$ as compared to UGT1A4 for the OLZ-4’-N-glucuronide but exhibited a higher $K_m$ as compared to UGT1A4 for both isomers of the OLZ-10-N-glucuronide. These data are consistent with the larger effect observed for the UGT2B10*2 allele on the rate of OLZ-4’-N-glucuronide formation and OLZ-4’-N-glucuronide kinetic parameters than that observed for either isomer of the OLZ-10-N-glucuronide and suggest that UGT2B10 is the most active hepatic UGT in terms of OLZ-4’-N-glucuronide formation.

The importance of UGT2B10 in OLZ glucuronidation was also implicated in experiments using the UGT1A4-specific inhibitor LTG. When LTG was used to inhibit UGT1A4 activity in
HLM, OLZ glucuronidation was decreased by 12%. While these data support an important role for UGT2B10 in the glucuronidation of OLZ, it is also likely that, unlike that observed for UGT1A4-over-expressing bacculosomes (32), LTG may not be the best inhibitor of OLZ in the cell line system utilized in the present study since excess LTG inhibited OLZ glucuronidation by only 41% in UGT1A4-over-expressing cell homogenates.

The UGT1A4<sup>24Pro<sup/>48Val</sup> variant exhibited significantly higher levels of OLZ-10-N-glucuronide formation (both isomers 1 and 2). While a discernable effect on OLZ-4’-N-glucuronide formation was not observed in vitro in UGT1A4-over-expressing cell homogenates, there was a significant trend towards increased formation of all three OLZ glucuronides both in terms of rate of formation as well as in kinetic parameters in HLM with increasing numbers of the UGT1A4*3 allele. These data are consistent with results from a recent study demonstrating a lower level of plasma OLZ in subjects with the more active detoxifying UGT1A4<sup>48Val</sup>-encoding *3 allele (36). While cell homogenates over-expressing the UGT1A4<sup>24Thr<sup/>48Leu</sup> variant exhibited a significant decrease in OLZ-10-N-glucuronide isomer 1 formation compared with wild-type UGT1A4<sup>24Pro<sup/>48Leu</sup> in over-expressing cell line homogenate studies, no effect on the formation of other OLZ glucuronides were observed in vitro and no association was observed between the formation of any of the three OLZ glucuronides and the UGT1A4*2 allele in HLM. Together, these data suggest that the UGT1A4 Leu48Val polymorphism is a prevalent UGT1A4 missense SNP that may be linked to altered hepatic OLZ glucuronidation activity.

The UGT2B10<sup>67Tyr</sup> variant has been described as a functional ‘knock-out’ of UGT2B10 enzyme activity (24, 421, 427, 430, 439, 440). The lack of in vitro activity against OLZ by the UGT2B10<sup>67Tyr</sup> variant is consistent with the observed association between the UGT2B10*2 allele and significant decreases in OLZ glucuronide formation in HLM.

The effect of the UGT2B10*2 allele was most profound when comparing UGT2B10 (*1/*2)/UGT1A4 (*1/*1) HLM versus UGT2B10 (*1/*1)/UGT1A4 (*3/*3) HLM. A 2.7-3-fold
decrease in $V_{\text{max}}/K_M$ was observed for both isomers of the OLZ-10-$N$-glucuronide and a nearly 6-fold decrease in $V_{\text{max}}/K_M$ was observed for the OLZ-4'-$N$-glucuronide. In all cases, the single HLM exhibiting the UGT2B10 (*2/*2) genotype exhibited even lower activity. These data strongly implicate both the UGT1A4 Leu48Pro and UGT2B10 Asp67Tyr polymorphisms as modifiers of hepatic OLZ glucuronidation activity.

These polymorphisms likely contribute to the wide interindividual variability seen in OLZ plasma levels (20, 34-37). It is possible that individuals with these polymorphisms could be at a higher risk of OLZ treatment failure or development of side effects, most notably weight gain and metabolic dysfunction. Other studies have examined the effects of polymorphisms, enzyme induction, or enzyme inhibition of CYP1A2, responsible for formation of the 4'-$N$-desmethylOLZ metabolic product (35, 445, 446). However, as 4'-$N$-desmethylOLZ comprises only 1.5% of urine metabolites (254) and is a lesser plasma metabolite, the impact of variations in CYP1A2 is likely less significant than variations in the OLZ glucuronidation pathway in terms of overall OLZ clearance and metabolism.

**Analysis of CLZ Glucuronidation by HLM Stratified by UGT1A1 or UGT1A4 Genotypes**

The $K_M$ for formation of CLZ-5-$N$-glucuronide of HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype was slightly higher than that observed in cell lines over-expressing UGT1A4 in vitro (238 ± 5 compared to 173±19), suggesting a combined effect of both UGT1A4 and another UGT on CLZ-5-$N$-glucuronide formation may be possible. MRM analysis of UGT-over-expressing cell line glucuronidation reactions showed that UGT1A1 does catalyze the formation of the CLZ-5-$N$-glucuronide, but at levels that were below the ultra violet (UV) detection limit of the UPLC. This is supported by the HLM kinetic analyses, which showed a 1.1-fold ($p<0.05$) decrease in $V_{\text{max}}/K_M$ observed in HLM with the UGT1A1 (*28/*28)/UGT1A4 (*1/*1) genotype as compared to HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype.
The studies examining HLM glucuronidation rate when stratified by genotype showed a 1.4-fold decrease in CLZ-5-N-glucuronide formation in HLM with the UGT1A4 (*28/*28) genotype versus HLM with the UGT1A1 (*1/*1) genotype, although this difference was not significant \((p=0.1)\) nor was the trend of decreasing CLZ-5-N\(^+\)-glucuronide product formation with increasing numbers of the *28 allele \((p=0.09)\). However, it is likely that the contribution of another UGT such as UT1A1 to the formation of CLZ-5-N-glucuronide is low and that UGT1A4 is the major enzyme responsible for the formation of this glucuronide product.

The \(K_M\) for the CLZ-N\(^+\)-glucuronide of HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype was inbetween that observed in cell lines over-expressing UGT1A1 and UGT1A4 in vitro, but was closer to that reported in UGT1A1-over-expressing cell lines (differing by 245 \(\mu\)M compared to 457 \(\mu\)M, respectively) suggesting that UGT1A1 is the dominant UGT responsible for CLZ-N\(^+\)-glucuronide formation. Their dual role in the glucuronidation of CLZ to its CLZ-N\(^+\)-glucuronide product is supported by the \(~50\%\) lower effect of the UGT1A4 (*3/*3) allele on the rate of formation of CLZ-N\(^+\)-glucuronide compared to formation of CLZ-5-N-glucuronide. Also, in HLM with the UGT1A1 (*28/*28)/UGT1A4 (*1/*1) genotype, a 61% decrease in \(V_{max}\) was observed as compared to HLM with the UGT1A1 (*1/*1)/UGT1A4 (*1/*1) genotype. Individuals homozygous for the UGT1A1*28 allele reportedly have a \(70\%\) reduction in UGT1A1 expression (442). This, along with reports that UGT1A1 is expressed at \(2-5\)-fold higher levels in the liver than UT1A4, suggest that UGT1A1 is the major enzyme responsible for the formation of CLZ-N\(^+\)-glucuronide. Due to the instability of the CLZ-5-N-glucuronide in acidic conditions such as the urine (28), the CLZ-N\(^+\)-glucuronide is an important marker for CLZ metabolism and clearance.

The UGT1A4\(^{24\text{Pro}48\text{Val}}\) variant exhibited higher levels of CLZ-5-N-glucuronide, CLZ-N\(^+\)-glucuronide, overall CLZ-glucuronide formation. There was a trend towards increased formation of the two CLZ glucuronides both in terms of rate of formation as well as in kinetic parameters in
HLM with increasing numbers of the UGT1A4*3 allele. These data are consistent with other studies which support the increased detoxifying activity of the UGT1A4\textsuperscript{48Val}-encoding *3 allele (36). While cell homogenates over-expressing the UGT1A4\textsuperscript{24Thr/48Leu} variant exhibited a significant decrease in CLZ-5-\textit{N}-glucuronide and all CLZ-glucuronide formation compared with the wild-type UGT1A4\textsuperscript{24Pro/48Leu} in over-expressing cell line homogenate studies, no effect on the formation of other CLZ glucuronides was observed \textit{in vitro} and no association occurred between the formation of the two CLZ glucuronides and the UGT1A4*2 allele in HLM. Together, these data suggest that the UGT1A4 Leu48Val polymorphism is a prevalent UGT1A4 missense SNP that may be linked to altered hepatic CLZ glucuronidation activity.

Similarly, the UGT1A4\textsuperscript{24Pro/48Val} variant exhibited significantly higher levels of dmCLZ-5-\textit{N}-glucuronide formation and resulted in a significant trend both in rate of formation as well as in HLM kinetic parameters with increasing numbers of the UGT1A4*3 allele. dmCLZ is the active metabolite of CLZ, with circulating levels between 30-60\% of the CLZ dose and is thought to be responsible for some of the superior therapeutic efficacy compared to other SGA compounds. While dmCLZ is currently not FDA-approved for patient treatment, a number of academic articles have highlighted its potential as a psychototropic therapeutic choice. However, dmCLZ is associated with the development of agranulocytosis, a life-threatening adverse effect occurring in ~1\% of individuals that take CLZ. It is thought that a reactive intermediate if dmCLZ is toxic to leukocytes in a dose-dependent manner. It is possible that decreased dmCLZ clearance could contribute to the development of agranulocytosis and that clearance and inactivation of dmCLZ by UGTs may lower the risk of an individual developing agranulocytosis.

The UGT1A1 A(TA)\textsubscript{7}TAA promoter polymorphism decreases the rate of transcription initiation of the UGT1A1 gene and is associated with decreased substrate glucuronidation and
increased drug-induced toxicity (442, 443). The decreased in vitro glucuronidation activity against CLZ by the UGT1A1 *28 allele is consistent with the observed association between the UGT1A1*28 allele and decrease CLZ kinetic parameters in HLM.

The effect of the UGT1A1*28 allele was most profound when comparing UGT1A1 (*28/*28)/UGT1A4 (*1/*1) HLM versus UGT1A1 (*1/*1)/UGT1A4 (*3/*3) HLM – a 3.7-4.5-fold decrease in V_max/K_M was observed for the two CLZ glucuronides and a 4.2-fold decrease for all CLZ-glucuronide formation. These data strongly implicate both the UGT1A1 A(TA)7TAA and UGT1A4 Leu48Val polymorphisms as modifiers of hepatic CLZ glucuronidation activity.

These polymorphisms may contribute to the wide interindividual variability in CLZ plasma levels seen clinically (28, 30, 31). It is possible that these polymorphisms contribute to CLZ treatment efficacy or the risk of developing adverse effects, most notably weight gain, metabolic dysfunction, and agranulocytosis.

Conclusions

The data presented in this study suggest that the UGT1A1 A(TA)7TAA, UGT1A448Val and UGT2B1067Tyr variants significantly alter OLZ, CLZ, and dmCLZ glucuronidation in vitro and could be important in determining interindividual differences in OLZ, CLZ, and dmCLZ metabolism in vivo. Other factors such as drug-induced UGT-gene expression, cigarette smoking, age, and gender also contribute to interindividual differences in drug metabolism. Therapeutic drug monitoring (TDM) is a costly method used to ensure plasma drug levels are within the therapeutic window and accounts for all causes of differences in drug metabolism. However, TDM cannot provide information regarding therapy efficacy and risk of adverse effects for a
given patient before starting a therapy. The results presented in this chapter could lead to new screening strategies to help determine dosing and patient response to OLZ or CLZ before starting treatment of schizophrenia, bipolar disorder, or treatment-resistant depression.
Chapter 6

FUTURE DIRECTIONS

Phenotype:Genotype Study of Second-Generation Antipsychotic Metabolite Levels, Patient Response, and UGT Genotype

In the previous chapter, the UGT1A4 (*3/*3)/UGT2B10 (*1/*1) genotype was associated with significantly higher metabolism of OLZ, CLZ, and dmCLZ, whereas the UGT1A4 (*1/*1)/UGT2B10 (*2/*2) genotype was associated with significantly lower activity against OLZ in HLM. Polymorphisms in these UGTs may influence SGA metabolism and clearance in vivo, which would alter plasma concentrations of these drugs and may translate to increased or decreased efficacy and risk of adverse effects. In this chapter, the ongoing clinical study examining a phenotype:genotype relationship between UGT variant alleles and SGA plasma concentrations, levels of plasma and urine SGA metabolites, and metabolic markers in volunteers will be outlined.

Introduction

Second generation antipsychotics (SGAs) are clinically utilized and FDA approved for treatment of schizophrenia (447), bipolar disorder (399, 400), and treatment-resistant depression in combination with fluoxetine. Although SGAs have lower discontinuation rate and a greater reduction in psychopathology as compared to FGAs (404, 448), they are associated with varying degrees of weight gain in patients compared to patients on FGAs or the normal population (404-409), contributing to increased metabolic dysfunction, dyslipidemia, overweight/obesity, type II diabetes mellitus, heart disease and mortality (410, 411).
OLZ and CLZ are associated with severe weight gain, whereas risperidone and quetiapine are associated with moderate weight gain, and ziprasidone and aripiprazole are associated with modest weight gain. These differences are thought to be accounted for, in part, by the receptor binding and affinity of the different SGAs towards 5-HT, DA, and histaminergic receptors. Efficacy trials of the recently released SGAs iloperidone and paliperidone report that they are associated with weight gain; however, it is unclear how much weight gain, metabolic syndrome, diabetes and heart disease will be associated with their chronic administration compared to other SGAs on the market.

SGA plasma levels vary widely among individuals regardless of dose. SGA plasma concentrations correlate with either efficacy or metabolic side effects rather than dose (21, 36, 38, 42, 449-451), indicating that interindividual variability in DMEs is important in the determination of an individual’s plasma levels and response. UGT enzymes form major and minor metabolites of SGAs (35, 254), such as OLZ-10-N-glucuronide, the major metabolite of OLZ in urine (254). CLZ undergoes extensive biotransformation, with only 3% of the administered dose excreted unchanged (17). CLZ can be converted to two primary CLZ-glucuronides and at least five secondary glucuronides in humans (26-28). Three of these metabolites account for approximately 30% of human CLZ metabolites (26); the remaining 70% has not yet been quantified (27). The previous chapters have shown that the variant allele, UGT1A4 *3, increases rates of metabolism of OLZ, CLZ, and its active metabolite dmCLZ. The UGT1A1 *28 variant decreases rates of CLZ metabolism, and the UGT2B10 *2 variant decreases rates of OLZ metabolism.

Since phase II glucuronidation by UGT enzymes constitutes a major metabolic pathway for these drugs, we believe that these variations in metabolism observed in vitro will translate to variations in plasma concentrations of SGAs, and their metabolites in vivo. The purpose of this study is to conduct a phenotype:genotype clinical study of the UGT 1A1, 1A4 and 2B10
genotypes with CLZ, dmCLZ, and OLZ plasma concentrations and the concentrations of plasma and urine SGA metabolites. These genotypes and their effect on plasma concentrations will then be examined for their relationship to the phenotypes of weight gain, metabolic syndrome, diabetes, dyslipidemia, and cardiovascular disease in human subjects.

**Materials and Methods**

**Study Design**

This study will have a cross-sectional design to test associations between polymorphisms of candidate genes (focusing initially on UGT1A1, UGT1A4 and UGT2B10) and SGA adverse effects (focusing initially on the severe weight-gain inducing SGAs OLZ and CLZ). A sample size of 750 is sufficient to represent the genotypes of interest with the lowest prevalence (see power/statistical analysis) and to account for subject withdrawal. Medical chart reviews will be performed to determine history of metabolic adverse effects of SGAs, including weight gain, type II diabetes, hyperglycemia, impaired oral glucose tolerance, dyslipidemia, metabolic syndrome, and heart disease.

**Population to be Studied**

The study population consists of 750 volunteers taking OLZ or CLZ. The goal will be to recruit 375 males and 375 females and to recruit a racial/ethnic representation equivalent to our catchment area. Based on recent census data, the surrounding area of Dauphin County is 17.5% African American, 2.7% Asian, 6.3% Hispanic or Latino, and 72.6% Caucasian while Harrisburg, PA is 54.8% African American, 2.8% Asian, 11.7% Hispanic or Latino, and 31.7% Caucasian. The minimum participant age will be 18 years old as patient populations taking OLZ and CLZ
will rarely include subjects younger than this age. Furthermore SGAs are currently FDA-approved for use in adult populations only.

**Inclusion and Exclusion Criteria**

Eligible individuals will be at least 18 years old with no history of confounding medical disorders, such as liver or kidney impairment or hepatic or renal blood flow insufficiency. Individuals must be able to give their consent to participate in the study. Subjects should not concurrently be taking medications that interfere with the metabolism and clearance of OLZ or CLZ, such as carbamazepine, fluvoxamine and omeprazole (Table 6-1).

**Table 6-1 Inclusion and exclusion criteria**

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Aged at least 18 years</td>
<td>• Presence of any medical disorder that may confound the assessment of relevant biologic measures, including:</td>
</tr>
<tr>
<td>• Able to give informed consent</td>
<td>• significant organ system dysfunction (kidney, liver)</td>
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<tr>
<td>• Taking OLZ or CLZ</td>
<td>• endocrine disease</td>
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<tr>
<td></td>
<td>• blood clots</td>
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<td></td>
<td>• clinically significant anemia</td>
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<td>• acute infection</td>
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<td></td>
<td>• Subjects taking certain prescription medications that effect the metabolism of OLZ or CLZ. Examples of these medications include: carbamazepine, fluvoxamine, omeprazole, rifampin, and activated charcoal.</td>
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**Recruitment and Consent Process**

Subjects will be recruited from The Pennsylvania Psychiatric Institute (PPI) and Milton S. Hershey Medical Center inpatient populations, but may be recruited through institutional satellite clinics in the surrounding area to attract a racially/ethnically diverse population. Eligible individuals will be identified by their physicians and informed of the study. If they indicate interest, the physician will inform one of the investigators associated with this protocol that is not primarily responsible for the care of the patient, who will read a standard script to the potential subject to inform them in layman’s terms about the study (Appendix). The script outlines the purpose of the study, the requirements of the subjects, the potential benefits to them and society, compensation, and explicitly states that they are not required to participate in the study as a part of their care, but can participate by choice. For individuals that meet the inclusion and exclusion criteria, a consent form that clearly explains the goals and procedures of the study will be given to the individual. Risks as well as procedures to protect the subjects from harm will be explained in lay word. Informed consent will be obtained after the subject has had time to read the consent form and have all questions answered (Appendix). Subjects will be given a copy of the consent form to take home.

**Study Procedures**

**General Procedures.**

Volunteers will be either inpatient or outpatient psychiatric patients. During a routine medical visit or stay, subjects will be asked to take their prescribed dose of the drug of interest. Following a routinely scheduled blood draw, a second tube of blood (~20 cc) will be obtained from 1) patients in order to measure serum concentrations of OLZ and CLZ metabolites and to 2) perform genotyping of the UGT genes by utilizing isolated lymphocytes from the subjects blood
and designed genotyping determination assays. Urine (~50cc) will be collected non-invasively to determine the levels of drug and drug metabolites in the urine. Inpatients and outpatients at both institutions routinely have venipunctures during their stay or visit. Five subjects will undergo a time-course study to determine how OLZ and CLZ plasma and urine concentrations fluctuate between doses and to determine the optimal blood draw time post-dose as this is the standard sample size for these types of studies. Samples will be taken at 1, 2, 4, 8, 12, and 24 hours post-dose for CLZ and dmCLZ (t1/2 approximately 8h) and 1, 2, 4, 8, 16, 32, and 48 hours post-dose for OLZ (t1/2 approximately 21-24h) (~10 cc draw each time) and perform a 24 hour urine collection. Time course blood draws will follow a regularly scheduled blood draw as much as is possible. A line will be used rather than multiple needle sticks. All identifying patient information associated with the samples will be de-identified and stored on computers that are password protected in the laboratory and office suites of Dr. Philip Lazarus (T3115). Only the investigators associated with this proposal will have access to identifying patient information for the purposes of chart review. The data will be de-identified 6 years after the study completion per federal guidelines.

**Experimental.**

Lymphocytes will be isolated from collected blood specimens for genotyping, with genomic DNA purified from lymphocytes using standard techniques within the Lazarus lab. All samples will be blinded to researchers during genotyping experiments. Standard real-time PCR will be the genotyping method of choice, a method that has been used extensively in our laboratory. After the genotype determination assays are performed, alternative methods, including DNA sequencing or an alternative genotyping assay, will be utilized for validation of the primary assays. Quality control of our genotyping data will be performed by randomly re-testing 5% of
the samples. UGT genotype will be compared to drug and drug metabolite plasma concentrations. Plasma drug and plasma and urinary drug metabolites will be quantified by UPLC/MS/MS using techniques similar to that described previously (6).

Medical chart reviews will be used to examine subject history of SGA adverse effects, such as hyperglycemia, insulin resistance, hypo- or hyper-insulinemia, weight gain, type II diabetes, dyslipidemia, cardiovascular disease, and treatment efficacy. Statistical analysis will be performed to examine if these adverse effects or efficacy correlate with certain UGT genotypes.

If the subject indicates agreement on the consent form, the researchers will store the leftover samples of that subject’s blood and urine. This leftover blood and urine can be used for other research in the future after this study is over. The leftover samples will be de-identified and labeled with a code number and stored in the locked laboratory and office suits of Dr. Lazarus at T3427.

Results

The protocol for the proposed study was submitted to the Penn State College of Medicine Institutional Review Board on October 5th, 2010, reviewed by the full board and approved December 15th 2010. Subject recruitment and sample acquisition is in process and ongoing.

Discussion

The currently approved protocol (Appendix) is for the study of UGT1A4 and UGT2B10 genotypes on OLZ plasma concentrations and metabolic side effect risk. There is much debate in the literature as to how to design a clinical pharmacogenetic study examining the effects of DME genotype on plasma drug concentrations and then on efficacy and risk of adverse effects. For
schizophrenic patients, it seems that the most the effect of the given UGT genotypes on measures of SGA adverse effects would be seen in a study population of antipsychotic-naive patients as they would not have previous weight gain from other antipsychotics. Furthermore, newly diagnosed patients should have a lower baseline BMI as schizophrenia is associated with weight gain as well.

The risk of weight gain on SGAs is highly variable depending on the study. According to Eli Lilly and Co., only 15% of patients on OLZ experience weight gain, where subsequently published, independent studies have reported incidences of weight gain up to 30%. Given that the highest allele frequency for the genotypes involved in OLZ metabolism is 11% in Caucasians, it is likely that UGT genotype is not the only contributing factor to OLZ-induced weight gain. Other pharmacokinetic factors involved in the absorption, metabolism and excretion of OLZ may contribute to the wide inter-individual variability seen in patients taking OLZ. Furthermore, interindividual differences in the expression of the UGTs involved, due to transcriptional, translational, or post-translational regulation, would affect the number of UGT enzymes expressed and the maximal UGT activity for an individual, and thus that individual’s drug plasma concentration.

Plasma concentrations of a drug are a measure of the concentrations seen at brain receptors of the drug (76). While SGA plasma concentrations are an accurate measure of brain concentrations, it is likely that receptor occupancy and drug-receptor rates of association and dissociation are important mediators of efficacy and adverse effects. Thus, pharmacodynamic factors likely contribute to efficacy of SGAs and weight gain risk as well as pharmacokinetic factors. PET scanning of receptor occupancy is an effective technique to monitor receptor activation by antipsychotics. Thus, it is likely that a combination of therapeutic drug monitoring and PET imaging may be a better way to predict and/or monitor efficacy in patients. However, this is expensive and does not guarantee success. Furthermore, patients whom chronically take
antipsychotics have differences in receptor expression and sensitivity over time. There are theories proposing that SGA action at extra-neuronal tissues may have an effect on glucose metabolism or overall metabolic rate (178, 180, 182, 183). Developing a genotype screening test that involves multiple pharmacokinetic and dynamic targets would help personalize a treatment to optimize efficacy and minimize adverse effects.

**Conclusions**

In this chapter an IRB-reviewed and approved ongoing clinical study examining the relationship between OLZ plasma levels, adverse effects, and UGT genotype was described. This study will likely be expanded to include other SGAs, including CLZ and dmCLZ.
Chapter 7

CONCLUSIONS

These studies investigated the influence of UGT genotype on OLZ, CLZ, and dmCLZ glucuronidation using two in vitro models: a HEK293 UGT-overexpressing cell line system and human liver microsomes. Additionally, in vivo ongoing research was described investigating the influence of UGT genotype on OLZ, CLZ, and dmCLZ plasma concentrations, efficacy, and tolerability. Collectively, the findings are as follows: 1) The SGAs OLZ, CLZ, and dmCLZ are substrates of UGTs, 2) Variations in OLZ, CLZ, and dmCLZ glucuronidation occur according to genotypes of specific UGT enzymes, and 3) these variations could contribute to the dramatic interindividual variability in plasma concentrations of these drugs in vivo, and may be one factor that determines efficacy or risk of adverse effects.

UGT Involvement in the Metabolism of the Severe-Weight Inducing SGAs

Previous to the present studies UGT metabolism of the SGAs OLZ, CLZ and dmCLZ was largely uncharacterized. While it was known that the major human metabolite of OLZ was the OLZ-10-N-glucuronide, most in vitro drug metabolism studies focused on CYP450-catalyzed metabolism of OLZ. As a result, most in vivo pharmacogenetic studies focused on CYP450, rather than UGT, genotype. The same is true for CLZ and its active metabolite dmCLZ. In Chapter 1 of this dissertation the results show that UGTs 1A4 and 2B10 are active against OLZ, UGTs 1A1 and 1A4, and to a lesser extent UGTs 1A3 and 2B10 are active against CLZ. UGT1A4 is active against the active CLZ metabolite dmCLZ. All of these UGTs are expressed in liver, the major site of drug metabolism in vivo. UGTs 1A1, 1A3, and 1A4 are expressed in the
digestive tract, which is the location of drug absorption. Thus, both hepatic and extrahepatic UGT metabolism at target tissues and sites of absorption may be important in the clearance of these drugs.

There is emerging evidence of target-tissue drug metabolism and its importance to drug efficacy and side effects. CYP450 enzymes are expressed in the brain and have a role in crossing the blood-brain barrier and in brain drug metabolism by regulating drugs' influx and by modulating blood-flow regulation (452-454). UGTs are expressed at relatively low concentrations in the brain and higher in the brain endothelium (455, 456). Thus, both hepatic and brain-tissue metabolism of CLZ may be important to its clearance and inactivation.

In the liver, UGT2B10 is the most highly expressed of the UGTs active against the severe-weight gain inducing SGAs, followed by UGT1A1, 1A4 and 1A3 ((457), Figure 7-1).

Figure 7-1 Tissue-specific expression of UGT enzymes by real-time PCR. The units of the y-axis represent the UGT copy number x 10^4 normalized to GAPDH (457).
However, it is unclear if mRNA expression of these UGTs is correlated with protein levels and activity. As discussed in Chapter 2, there are a number of gene, transcriptional, translational, and post-transcriptional modifications that could affect UGT protein levels and activity in a given tissue.

In general, UGT activity against SGAs and other psychotopic drugs is largely uncharacterized compared to the CYP450s despite their importance to drug metabolism and clearance. This is likely due to the lag time between the characterization of CYP450s due to the cross-reactivity of UGTs towards substrates and a relative lack of specific antibody probes (455). However, as shown in Chapter 3, there are a number of novel UGTs that likely have glucuronidation activity against other widely-prescribed SGAs and psychotropics, such as paliperidone. A preliminary UGT screen against paliperidone showed that the hepatically expressed UGT1A4 and extra-hepatic UGTs 1A4, 1A7, 1A8, and 1A9 exhibit glucuronidation activity against this substrate (Figure 7-2).
Given the dominance of UGTs in phase II drug metabolism, an important continuation of this work would be to characterize the UGTs that are active against other SGAs and psychotropic medications that are widely used, such as antidepressants, and the effect of UGT polymorphisms on enzyme activity against these substrates. The results of such studies could identify new targets that can be studied in vivo for their effects on drug metabolism and outcomes.
The Influence of Functional UGT Polymorphisms on Severe Weight-Gain Inducing SGA Glucuronidation

Despite the introduction of antipsychotics and progress over the past several decades in developing new classes of such medications that are presumably safer and more effective, the ability to treat mental illnesses remains clinically suboptimal (43). SGAs are effective in a subset of patients as evidenced by a discontinuation rate of approximately 74% (43, 286), produce partial responses, or are associated with significant weight gain and resultant sequelae including diabetes, dyslipidemia, and CVD that discourage adherence. As SGA plasma concentration are predictors of efficacy and drug toxicity (21, 31, 383-386, 458), it is important to elucidate the mechanisms contributing to the wide interindividual variation in SGA plasma concentrations.

UGT promoter and coding-region variants impact drug metabolism in vitro and in vivo (442). For example, the UGT1A1*28 polymorphism, in which patients have 7 “TA” repeats in the TATA box promoter region of UGT1A1 rather than 6 repeats, is associated with an approximately 30% decrease in expression of UGT1A1. The UGT1A1*28 allele is associated with decreased clearance of the chemotherapeutic agent irinotecan (SN-38 active metabolite) and a 3-4-fold increase in risk for irinotecan-associated adverse effects such as leukopenia and clinically-significant diarrhea (45). The UGT1A4 codon 48 polymorphism is associated with increased tamoxifen clearance in vitro using UGT overexpressing-cell lines and HLM (425, 459-461) and in vivo (462). The UGT2B10 codon 67 functional variant is associated with decreased clearance of nicotine and tobacco-specific nitrosamines (TSNAs) in UGT overexpressing-cell lines and HLM in vitro and this genotype correlates with TSNA-glucuronide clearance as evidenced in patient urine (24, 439, 440).

In chapter 4 of this dissertation, the functional variants of UGT1A4 and UGT2B10 were examined in vitro. The UGT1A4 codon 24 variant (Proline to Threonine amino acid conversion), had an effect on UGT1A4 formation of the tertiary N-glucuronide products OLZ-10-N-
glucuronide isomer 1, the CLZ-5′-N-glucuronide, and the dmCLZ-5′-N-glucuronide (Table 4-1, Table 4-2, and Table 4-3, respectively). This effect, which ranged between 1.1-3.4 fold for the different products, seems to be mediated mainly by an increase in the K_M. The K_M is an inverse measure of enzyme affinity for the drug, with a lower value representing a higher enzyme affinity for a given substrate. Thus, it seems that the codon 24 polymorphism alters the UGT1A4 substrate binding site in a way that decreases its ability to bind the substrate. In the codon 24 variant, a neutral proline amino acid is substituted for a polar threonine amino acid. This change could contribute to decreasing the binding site affinity for SGAs. When the effects of the UGT1A4 codon 24 variant were examined in HLMs, no significant effect was observed on HLM glucuronidation activity or kinetics against OLZ, CLZ, or dmCLZ (Figure 5-1, Figure 5-2). These results are similar to previously published research examining the UGT1A4 codon 24 variant against other substrates (419, 463).

The UGT1A4 codon 48 functional variant (Leucine to Valine change) was first described with an allelic prevalence of 9% in Caucasians (419). Since then, it has been shown in numerous studies to increase the glucuronidation of many endogenous and exogenous substrates in vitro and in vivo via an increase in enzymatic activity of the UGT1A4 enzyme (419, 441, 442, 462). In Chapter 4 of this dissertation, the UGT1A4 codon 48 variant was shown to change UGT1A4 kinetics compared to wild-type UGT1A4 in HEK 293 over-expressing cell lines for all metabolic products of OLZ, CLZ, and dmCLZ (Table 4-1, Table 4-2, Table 4-3). Overall, the codon 48 variant increased glucuronidation efficiency of the UGT1A4 enzyme as evidenced by an increase in V_max/K_M via a 1.7-4-fold increase in V_max or a 2.5-fold decrease in K_M. Valine and Leucine are both nonpolar, neutral amino acids with valine having a slightly higher hydropathy index. The side chain of leucine is one carbon longer than that of valine, potentially allowing substrates to fit into the active site of UGT1A4 with greater ease by minimizing steric effects. As shown in Chapter 5, the codon 48 variant increased glucuronidation product formation for all three SGAs
by ~2-3 fold and improved HLM glucuronidation efficiency as measured by $V_{\text{max}}/K_{M}$ as well. These changes are mediated by approximately a 2-3-fold increase in $V_{\text{max}}$ as well as a reduction in $K_{M}$. For OLZ, CLZ, and dmCLZ, the changes observed in kinetic parameters with the UGT1A4*48V variant in overexpressing cell lines were similar to those observed with an increasing number of the UGT1A4 *3 allele in HLM. For all three drugs, UGT1A4 mediates 40-100% each glucuronide product formation, indicating that the UGT1A4*48V variant may be an important pharmacogenetic target in vivo.

The UGT2B10 codon 67 variant (Aspartic Acid to Tyrosine change) has an allelic prevalence of 10-11% in Caucasians (421). When OLZ glucuronidation was examined using the UGT2B10*67Y-overexpressing-cell line in Chapter 4, this variant resulted in a complete loss of glucuronidation activity (Table 4-1). This result is due to a decrease in enzyme activity and not differences in UGT2B10- and UGT2B10*67Y-overexpressing-cell line mRNA expression or protein levels as evidenced by real-time PCR and Western blot analysis (Figure 3-5, Figure 4-1). Previously published reports confirm that the codon 67 variant results in a loss of UGT2B10 activity (24, 427, 439). In HLM, the UGT2B10*2 allele was associated with a 50-65% reduction in OLZ-glucuronide formation, which agrees well with the UGT2B10*67Y-overexpressing cell line observed kinetic activity. Studies using the UGT1A4-specific inhibitor LTG confirm the importance of UGT2B10 in OLZ metabolism by HLMs. This is the first report of UGT2B10 glucuronidating OLZ. Previous to these studies, it was known that the major metabolic pathway for OLZ was via glucuronidation and that UGT1A4 was active against OLZ (33, 254, 398). Given the relatively higher levels of UGT2B10 expression in the liver compared to UGT1A4 (305, 457) and the relative contribution of UGT2B10 to HLM glucuronidation of OLZ, it seems that UGT2B10 is the major UGT enzyme responsible for OLZ glucuronidation. This may translate to the UGT2B10 genotype serving as a predictor of OLZ plasma concentrations in patients.
The UGT1A1*28 polymorphism in relationship to CLZ glucuronidation was examined in HLM in Chapter 5 of this dissertation. In both glucuronidation rate and kinetic studies stratifying HLM by UGT1A1 genotype, the *28 polymorphism was a predictor of CLZ glucuronidation and resulted in a 60-70% reduction in the formation of the CLZ-N\(^+\)-glucuronide (Figure 5-5). The UGT1A1*28 polymorphism decreased transcription of the UGT1A1 gene; individuals homozygous for the *28 polymorphism expressed approximately 30% less UGT1A1 than normal homozygous individuals. This difference resulted in decreased drug glucuronidation and clearance and was associated with drug toxicities (442). In Chapter 5, the UGT1A1*28 polymorphism was associated with a trend toward decreased CLZ-N\(^+\)-glucuronide formation and decreased enzyme velocity (\(V_{\text{max}}\)) in HLM homozygous variant for the *28 allele. The CLZ-N\(^+\)-glucuronide is the major glucuronide metabolite identified in urine, probably due to its stability in acidic conditions (28). Thus, the UGT1A1 *28 polymorphism may predict CLZ metabolism in patients taking this drug.

A Phenotype:Genotype Clinical Study of Severe-Weight Gain Inducing SGA Plasma Levels, Side Effects, and UGT Genotype

The studies in Chapters 3-5 of this dissertation identified the UGTs active against the severe weight-gain inducing SGAs and characterized the impact of common functional variants on glucuronidation rate and enzyme kinetics in overexpressing cell lines and in HLM. These studies laid the ground work for a clinical study examining the effects of UGT genotypes on plasma concentrations of OLZ, CLZ, and dmCLZ, and their glucuronide metabolites, and how these concentrations correlate with therapeutic efficacy or adverse effects of these drugs. The design and IRB-approval for this study were outlined in Chapter 6 and samples are currently being procured. Such a study could provide new pharmacogenetic targets that ultimately will
translate to a genotype screening test that may help determine the most efficacious SGA drug for an individual with minimal risk for adverse effects before initiating SGA therapy. Furthermore, identifying the UGTs active against OLZ, CLZ, and dmCLZ might allow for targeted induction or inhibition of these UGTs using non competitive inhibitors of UGT to alter drug metabolism (464). However, this strategy is complicated by the similar substrate-specificity shared by many UGTs and the importance of UGTs in the metabolism of other endogenous and exogenous substrates and other genes.

Previous studies underestimated or overlooked the importance of UGTs in the metabolism of SGAs. This neglect may be due in part to the lack of sensitive and specific tools to quantify the UGTs compared to the CYP450s. Since the primary means of treating mental illness is through pharmacological intervention, understanding how these drugs are inactivated and cleared from the body is an important component of patient care. Understanding SGA pharmacogenetics is an important step in providing individualized medicine, where pharmacologic response can be predicted using genotyping technology or genetic information as biomarkers. Currently, one pharmacogenetic test has been approved by the FDA for clinical use (465, 466): The AmpliChip CYP450 Test uses Affymetrix microarray-based genotyping to assay for 20 CYP2D6 alleles, 7 CYP2D6 duplications, and 3 CYP2C19 alleles. The test includes software with an algorithm to predict metabolizing phenotypes (PM, IM, EM, and UM) based on the identified alleles. However, there remains insufficient evidence to establish appropriate uses of this screening test and to judge what impact it will have on SGA therapy.

Despite progress in pharmacogenetic research and its clinical applications, the potential impact of pharmacogenetics in psychiatry remains to be realized. There are formidable obstacles in translating genetic targets to the clinic, such as obtaining a clear understanding of how genetic factors influence responses to psychotropic medication. It is difficult to recruit sufficient numbers of patients to evaluate the influence of genetic polymorphisms on SGA efficacy and adverse
effect risk. Responses to psychotropic medications depend on a number of different pathways involved in their mechanism of action, metabolism and excretion. Thus, multiple genetic determinants in distinct and converging molecular pathways may independently and interactively contribute to an individual’s response to a particular therapy. In addition, multiple environmental factors contribute to large variability among patients in drug response, including diet, other medications, demographic factors, other disease states, and age. These and future pharmacogenetic studies continue to hold potential to improve the care of psychiatric patients.
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Appendix: Consent Forms

Single Blood Draw Consent Form

CONSENT FOR RESEARCH
Penn State College of Medicine
The Milton S. Hershey Medical Center

Title of Project: The role of UGT1A4 and UGT2B10 genotypes on olanzapine metabolism and patient response.

Principal Investigator: Philip Lazarus, Ph.D.

Other Investigators: Kathryn K. Erickson, B.S., Alan Gelenberg, M.D., Edward Bixler, Ph.D.; Ahmad Hameed, M.D.

Telephone Numbers: Weekdays: 8:00 a.m. to 5:00 p.m. (717) 531-5734. Evenings/Weekends/Holidays: (717) 531-8521. Ask for the Psychiatry doctor on 24-hour call.

Participant’s Printed Name: _____________________________

We are asking you to be in a research study.
You do not have to be in the research study. If you agree to be in the research study, you can quit at any time.
This form gives you information about this research study. Please ask questions about anything that is unclear to you.
Please take your time to make your choice.
1. Why is this research study being done?

We are asking you to be in this research because you are taking olanzapine and you have expressed interest in participating in this study. This research is being done to find out the effects of UDP-glucuronosyltransferases (UGT1A4 and 2B10 enzyme genetic polymorphisms on metabolism of olanzapine (Zyprexa®). “Metabolism” is the process through which your body removes chemicals, such as drugs. “UGTs” are a family of enzymes that help inactivate and remove compounds, such as drugs, from the body. “Enzymes” are proteins in the body that increase the rate of normally-occurring chemical reactions in your body. UGT1A4 and UGT2B10, two different types of UGT enzymes found in the body, have been found to metabolize olanzapine. Olanzapine is a member of a class of drugs called ‘second generation antipsychotics’ and is an FDA-approved drug to treat schizophrenia and other psychiatric disorders. In some individuals, these drugs have been shown to cause weight gain and in some cases diabetes with long-term treatment (at least >2 month). Recent studies have correlated higher blood levels of olanzapine with these long term effects (weight gain, diabetes). Our laboratory has shown that genetic polymorphisms, or variations, in the UGT1A4 and UGT2B10 genes result in faster or slower metabolism of olanzapine. These differences in speed of olanzapine metabolism may contribute to inter-individual variability in olanzapine blood levels and predispose an individual to weight gain. The purpose of this research is to see whether or not genetic polymorphisms in the UGT1A4 and UGT2B10 genes effect plasma levels of olanzapine.

Approximately 750 people (375 Males, 375 Females) are expected to take part at the Hershey Medical Center and Pennsylvania Psychiatric Institute.

2. What will happen in this research study?

If you are eligible to take part in this research study, an authorized individual will talk with you in private about the study and answer any questions you might have. This is to ensure that you completely understand your time commitment and the purpose and risks of this research.

If you decide to take part in this research study, you will be asked to take your olanzapine as you normally do. About 1-8 hours after you take your olanzapine right after a regularly scheduled blood draw, a small (~20cc or 1-2 tablespoons) blood sample will be taken to determine your type of UGT1A4 and UGT2B10 gene and to measure plasma levels of olanzapine and its metabolites. Also, we will collect urine (~50cc or 3-4 tablespoons) to look at levels of olanzapine and its metabolites. The results of these research tests will not have an effect on your care. Neither you, nor your doctor will receive
the results of these research tests, nor will the results be put in your medical record. We will review your medical chart to see if you have any history of metabolic side effects described by the drug manufacturer, such as weight gain, high blood sugar, or diabetes, and to determine the effectiveness of your olanzapine treatment.

If you give permission, a sample of plasma and urine to be kept indefinitely in a locked freezer for future lab tests. For information about this optional storage, see the end of this form. This sample will be given a new identification number after the conclusion of the study.

3. What are the risks and possible discomforts from being in this research study?

**Blood sampling:**
The discomfort associated with removing blood by venipuncture (by needle from a vein) is a slight pinch or pinprick when the sterile needle enters the skin.

The risks include:
- mild discomfort and/or a black and blue mark at the site of puncture
- Less common risks include:
- A small blood clot
- infection or bleeding at the puncture site
- Rare risks or discomforts include:
- dizziness or fainting during the procedure.

**Urine collection:**
There are no risks associated with urine collection.

There is a risk of loss of confidentiality if your medical information or your identity are obtained by someone other than the investigators, but precautions will be taken to prevent this from happening.

4. What are the possible benefits from being in this research study?

4a. What are the possible benefits to me?

You will not benefit from this research study.

4b. What are the possible benefits to others?

The results of this research may guide the future use of olanzapine for the treatment of disorders such as schizophrenia, bipolar disorder, and treatment-resistant depression.
5. What other options are available instead of being in this research study?

You may choose not to be in this research study.

6. How long will I take part in this research study?

If you agree to take part in this study, you will be in the research study for approximately 20 minutes.

7. How will you protect my privacy and confidentiality if I decide to take part in this research study?

7a. What are the measures taken to protect my privacy and confidentiality?

In our research files at The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU) we will include these identifiers: medical record number, name, and dates.

- A list that matches your name and medical record number with your coded sample ID number will be kept on a password protected computer file in Dr. Lazarus’ office.
- Your research records will be labeled with your code number and will be kept in a safe area in Dr. Lazarus’ research office and laboratory suites.
- Your research samples will be labeled with a code number and will be stored in Dr. Lazarus’ locked laboratory freezer at Hershey Medical Center.

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

7b. How will my identifiable health information be used?

This section is about health information that can be traced to you.

- If you give your consent, health information that can be traced to you will be collected for this research study. If you do not want us to use your health information, you should not be in this research.
- Health information is protected by law as explained in the HMC Privacy Notice. If you have not received this notice, please request a copy from the researcher.
- At HMC/PSU your information will only be used or shared as explained in this consent form or when required by law.
- Your permission for the use, storage, and sharing of your health information will continue indefinitely.
• If you say yes at this time but change your mind later you can tell us to stop using and sharing health information that can be traced to you. You must do this in writing. Write to inform Dr. Lazarus that you are withdrawing from the research study. His mailing address is: 500 University Dr, CH69, Hershey, PA 17033.

• If you change your mind and tell us to stop using your information:
  o We will stop collecting health information about you for this research study, except when the law allows us to do so.
  o We may continue using and sharing your information that we already have if it is necessary for the safety and scientific soundness of the research study.
  o We cannot take back anything we have already done or any information we have already shared.
  o We will keep our records of any care that we provided to you as long as the law requires.

• The research team may use the following health information.
  o Blood and urine samples and related tests
  o Information from your medical records related to the research study.
  o New health information from tests, procedures, visits, interviews, or forms filled out as part of this research study.

The following people/groups within HMC/PSU may use your health information and share it with these groups for this research study.

• The principal investigator, Dr. Philip Lazarus
• The research team for this investigation
• The HMC/PSU Institutional Review Board
• The HMC/PSU Human Subjects Protection Office
• The HMC/PSU Research Quality Assurance Office

The above people/groups may share your health information with the following people/groups outside HMC/PSU for this research study.

• The Office for Human Research Protections in the U. S. Department of Health and Human Services

These groups may also review and/or copy your original PSU/HMC records while looking at the results of the research study. It is possible that some of the other people/groups who receive your health information may not be required by Federal privacy laws to protect your information.
8. What are the costs of taking part in this research study?

8a. What will I have to pay for if I take part in this research study?

There is no cost to you for taking part in this research study, nor will you incur extra costs for taking part. If you have any questions about costs and insurance, ask the research study doctor or a member of the research team.

8b. What happens if I am injured as a result of taking part in this research study?

It is possible that you could develop complications or injuries as a result of being in this research study. If you experience a side effect or injury and emergency medical treatment is required, seek treatment immediately at any medical facility. If you experience a side effect or injury and you believe that emergency treatment is not necessary, you should contact Dr. Alan Gelenberg (717) 531-8516 as soon as possible and he/she will arrange for medical treatment.

HMC/PSU compensation for injury

- There are no plans for HMC/PSU to provide financial compensation or free medical treatment for research-related injury.
- If an injury happens, medical treatment is available at the usual charge.
- Costs will be charged to your insurance carrier or to you.
- Some insurance companies may not cover costs associated with research injuries.
- If these costs are not covered by your insurance, they will be your responsibility.

When you sign this form you are not giving up any legal right to seek compensation for injury.

9. Will I be paid to take part in this research study?

You will not receive any payment or compensation for being in this research study. It is possible that your samples and information may be used to develop products and tests that could be patented and licensed. There are no plans to provide financial compensation to you should this occur.
10. Who is paying for this research study?

The investigators are using funds from the Penn State College of Medicine Department of Pharmacology to support this research study.

11. What are my rights if I take part in this research study?

Taking part in this research study is voluntary.

- If you choose to take part in this research, your major responsibilities will include providing blood and urine samples.
- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

During the course of the research you will be provided with any new information that may affect your decision to continue participating in this research.

12. If I have questions or concerns about this research study, whom should I call?

Please call the head of the research study (principal investigator), Dr. Philip Lazarus at 717-531-5734 if you:

- Have questions, complaints or concerns about the research.
- Believe you may have been harmed by being in the research study.

You may also contact the research protection advocate in the HMC Human Subjects Protection Office (HSPO) at 717-531-5687 if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- Have questions about your privacy and the use of your personal health information.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.
You may visit the HSPO’s web site at http://pennstatehershey.org/web/irb/ under participant information for:

- Information about your rights when you are in a research study;
- Information about the Institutional Review Board (IRB), a group of people who review the research to protect your rights; and
- Links to the federal regulations and information about the protection of people who are in research studies. If you do not have access to the internet, copies of these federal regulations are available by calling the HSPO at (717) 531-5687.
Signature and Consent/Permission to be in the Research

Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Participant: By signing this consent form, you indicate that you voluntarily choose to be in this research.

<table>
<thead>
<tr>
<th>Signature of Participant</th>
<th>Date</th>
<th>Time</th>
<th>Printed Name</th>
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Person Explaining the Research: Your signature below means that you have explained the research to the participant and have answered any questions he/she has about the research.

<table>
<thead>
<tr>
<th>Signature of person who explained this research</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
</table>

Printed Name

(Only approved investigators for this research may explain the research and obtain informed consent.)
In addition to the main part of the research study, there is another part of the research. You can be in the main part of the research without agreeing to be in this optional part.

Optional Storage of Tissue for Future Research

In the main part of this study, we are collecting blood and urine from you. If you agree, the researchers would like to store leftover samples of your blood and urine. This leftover blood and urine can be used for other research in the future after this study is over.

- These future studies may be helpful in understanding other metabolic side effects of psychiatric treatments.
- It is unlikely that these studies will have a direct benefit to you.
- The results of these tests will not have an effect on your care.
- Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record.
- Sometimes tissue is used for genetic research about diseases that are passed on in families. Even if your samples are used for this kind of research, the results will not be put in your health record.
- It is possible that your blood and urine might be used to develop products or tests that could be patented and licensed. There are no plans to provide financial compensation to you should this occur. If you have any questions, you should contact Dr. Philip Lazarus at (717) 531-5734.

Your leftover samples will be stripped of personal information labeled with a code number.

- These samples will be stored in the locked laboratory and office suits of Dr/ Lazarus at T3427.
- The length of time they will be used is unknown.
- You will be free to change your mind at any time.
- You should contact Dr. Philip Lazarus at (717) 531-5734 and let him know you wish to withdraw your permission for your blood and urine to be used for future research. Any unused blood and urine will be destroyed and not used for future research studies.
You should initial below to indicate what you want regarding the storage of your leftover blood and urine for future research studies.

a. Your samples may be stored and used for future research studies to learn about, prevent, treat or cure metabolic side effects of psychiatric treatments.

_____ Yes _____ No

b. Your samples may be stored and used for research about other health problems.

_____ Yes _____ No

c. Your samples may be shared with other investigators/groups without any identifying information.

_____ Yes _____ No

**Participant:** By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part of the research study.

Signature of Participant __________________________  Date ________  Time ________  Printed Name

**Person Explaining the Research:** Your signature below means that you have explained the optional part of the research to the participant and have answered any questions he/she has about the research.

Signature of person who explained this research __________________________  Date ________  Time ________

Printed Name
Title of Project: The role of UGT1A4 and UGT2B10 genotypes on olanzapine metabolism and patient response.

Principal Investigator: Philip Lazarus, Ph.D.

Other Investigators: Kathryn K. Erickson, B.S., Alan Gelenberg, M.D., Edward Bixler, Ph.D.; Ahmad Hameed, M.D.

Telephone Numbers: Weekdays: 8:00 a.m. to 5:00 p.m. (717) 531-5734. Evenings/Weekends/Holidays: (717) 531-8521. Ask for the Psychiatry doctor on 24-hour call.

Participant’s Printed Name: _____________________________

We are asking you to be in a research study.

You do not have to be in the research study. If you agree to be in the research study, you can quit at any time.

This form gives you information about this research study. Please ask questions about anything that is unclear to you.

Please take your time to make your choice.

1. Why is this research study being done?

   We are asking you to be in this research because you are taking olanzapine and you have expressed interest in participating in this study

   This research is being done to find out the effects of UDP-glucuronosyltransferases (UGT)1A4 and 2B10 enzyme genetic polymorphisms on metabolism of olanzapine (Zyprexa®). “Metabolism” is the
process through which your body removes chemicals, such as drugs. “UGTs” are a family of enzymes that help inactivate and remove compounds, such as drugs, from the body. “Enzymes” are proteins in the body that increase the rate of normally-occurring chemical reactions in your body. UGT1A4 and UGT2B10, two different types of UGT enzymes found in the body, have been found to metabolize olanzapine. Olanzapine is a member of a class of drugs called ‘second generation antipsychotics’ and is an FDA-approved drug to treat schizophrenia and other psychiatric disorders. In some individuals, these drugs have been shown to cause weight gain and in some cases diabetes with long-term treatment (at least >2 month). Recent studies have correlated higher blood levels of olanzapine with these long term effects (weight gain, diabetes). Our laboratory has shown that genetic polymorphisms, or variations, in the UGT1A4 and UGT2B10 genes result in faster or slower metabolism of olanzapine. These differences in speed of olanzapine metabolism may contribute to inter-individual variability in olanzapine blood levels and predispose an individual to weight gain. The purpose of this research is to see whether or not genetic polymorphisms in the UGT1A4 and UGT2B10 genes effect plasma levels of olanzapine.

Approximately 750 people (375 Males, 375 Females) are expected to take part at the Hershey Medical Center and Pennsylvania Psychiatric Institute. You are being asked to participate in a time-trial study to determine how blood levels of olanzapine and its metabolites change over 24 hours.

2. What will happen in this research study?

If you are eligible to take part in this research study, an authorized individual will talk with you in private about the study and answer any questions you might have. This is to ensure that you completely understand your time commitment and the purpose and risks of this research.

If you decide to take part in this research study, you will be asked to take your olanzapine as you normally do. We will draw a small (~10cc or 1 tablespoon) blood sample at 1, 2, 4, 8, 12, and 24 hours post-OLZ dose. An intravenous line will be put in a vein in your arm for the blood draws in order to reduce the number of needle sticks. The intravenous line will be kept in place by a protective tape. We will also ask you to do a 24-hour urine collection. The blood samples will be used to determine your type of UGT1A4 and UGT2B10 gene, to measure concentrations of olanzapine and its metabolites, and to determine how these concentrations change over a 24-hour period. We will also ask you to do a 24-hour urine collection to determine the levels of olanzapine and olanzapine metabolites. The results of these research tests will not have an effect on your care. Neither you, nor your doctor will receive the results of these research tests, nor will the results be put in your medical
record. We will review your medical chart to see if you have any history of metabolic side effects described by the drug manufacturer, such as weight gain, high blood sugar, or diabetes, and to determine the effectiveness of your olanzapine treatment.

If you give permission, a sample of plasma and urine to be kept indefinitely in a locked freezer for future lab tests. For information about this optional storage, see the end of this form. This sample will be given a new identification number after the conclusion of the study.

3. What are the risks and possible discomforts from being in this research study?

**Blood sampling:**
The discomfort associated with removing blood by venipuncture (by needle from a vein) is a slight pinch or pinprick when the sterile needle enters the skin.

The risks include:

- mild discomfort and/or a black and blue mark at the site of puncture
- Less common risks include:
  - A small blood clot
  - infection or bleeding at the puncture site
- Rare risks or discomforts include:
  - dizziness or fainting during the procedure.

**Urine collection:**
There are no risks associated with urine collection.

There is a risk of loss of confidentiality if your medical information or your identity are obtained by someone other than the investigators, but precautions will be taken to prevent this from happening.

4. What are the possible benefits from being in this research study?

4a. What are the possible benefits to me?

You will not benefit from this research study.

4b. What are the possible benefits to others?

The results of this research may guide the future use of olanzapine for the treatment of disorders such as schizophrenia, bipolar disorder, and treatment-resistant depression.
5. **What other options are available instead of being in this research study?**

You may choose not to be in this research study.

6. **How long will I take part in this research study?**

If you agree to take part in this study, you will be in the research study for approximately 24 hours.

7. **How will you protect my privacy and confidentiality if I decide to take part in this research study?**

   **7a. What are the measures taken to protect my privacy and confidentiality?**

   In our research files at The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU) we will include these identifiers: medical record number, name, and dates:

   - A list that matches your name and medical record number with your coded sample ID number will be kept on a password protected computer file in Dr. Lazarus’ office.
   - Your research records will be labeled with your code number and will be kept in a safe area in Dr. Lazarus’ research office and laboratory suites.
   - Your research samples will be labeled with a code number and will be stored in Dr. Lazarus’ locked laboratory freezer at Hershey Medical Center.

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

   **7b. How will my identifiable health information be used?**

   This section is about health information that can be traced to you:

   - If you give your consent, health information that can be traced to you will be collected for this research study. If you do not want us to use your health information, you should not be in this research.
   - Health information is protected by law as explained in the HMC Privacy Notice. If you have not received this notice, please request a copy from the researcher.
   - At HMC/PSU your information will only be used or shared as explained in this consent form or when required by law.
   - Your permission for the use, storage, and sharing of your health information will continue indefinitely.
If you say yes at this time but change your mind later you can tell us to stop using and sharing health information that can be traced to you. You must do this in writing. Write to inform Dr. Lazarus that you are withdrawing from the research study. His mailing address is: 500 University Dr, CH69, Hershey, PA 17033.

If you change your mind and tell us to stop using your information:
- We will stop collecting health information about you for this research study, except when the law allows us to do so.
- We may continue using and sharing your information that we already have if it is necessary for the safety and scientific soundness of the research study.
- We cannot take back anything we have already done or any information we have already shared.
- We will keep our records of any care that we provided to you as long as the law requires.

The research team may use the following health information.
- Blood and urine samples and related tests
- Information from your medical records related to the research study.
- New health information from tests, procedures, visits, interviews, or forms filled out as part of this research study.

The following people/groups within HMC/PSU may use your health information and share it with these groups for this research study.

- The principal investigator, Dr. Philip Lazarus
- The research team for this investigation
- The HMC/PSU Institutional Review Board
- The HMC/PSU Human Subjects Protection Office
- The HMC/PSU Research Quality Assurance Office

The above people/groups may share your health information with the following people/groups outside HMC/PSU for this research study.

- The Office for Human Research Protections in the U. S. Department of Health and Human Services

These groups may also review and/or copy your original PSU/HMC records while looking at the results of the research study. It is possible that some of the other people/groups who receive your health information may not be required by Federal privacy laws to protect your information.
8. What are the costs of taking part in this research study?

8a. What will I have to pay for if I take part in this research study?

There is no cost to you for taking part in this research study, nor will you incur extra costs for taking part. If you have any questions about costs and insurance, ask the research study doctor or a member of the research team.

8b. What happens if I am injured as a result of taking part in this research study?

It is possible that you could develop complications or injuries as a result of being in this research study. If you experience a side effect or injury and emergency medical treatment is required, seek treatment immediately at any medical facility. If you experience a side effect or injury and you believe that emergency treatment is not necessary, you should contact Dr. Alan Gelenberg (717) 531-8516 as soon as possible and he/she will arrange for medical treatment.

HMC/PSU compensation for injury

- There are no plans for HMC/PSU to provide financial compensation or free medical treatment for research-related injury.
- If an injury happens, medical treatment is available at the usual charge.
- Costs will be charged to your insurance carrier or to you.
- Some insurance companies may not cover costs associated with research injuries.
- If these costs are not covered by your insurance, they will be your responsibility.

When you sign this form you are not giving up any legal right to seek compensation for injury.

10. Will I be paid to take part in this research study?

Once you complete the timed trial study, you will receive a $50 gift certificate. It is possible that your samples and information may be used to develop products and tests that could be patented and licensed. There are no plans to provide financial compensation to you should this occur.
10. **Who is paying for this research study?**

The investigators are using funds from the Penn State College of Medicine Department of Pharmacology to support this research study.

11. **What are my rights if I take part in this research study?**

Taking part in this research study is voluntary.

- If you choose to take part in this research, your major responsibilities will include providing blood and urine samples.
- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

During the course of the research you will be provided with any new information that may affect your decision to continue participating in this research.

12. **If I have questions or concerns about this research study, whom should I call?**

Please call the head of the research study (principal investigator), Dr. Philip Lazarus at 717-531-5734 if you:

- Have questions, complaints or concerns about the research.
- Believe you may have been harmed by being in the research study.

You may also contact the research protection advocate in the HMC Human Subjects Protection Office (HSPO) at 717-531-5687 if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- Have questions about your privacy and the use of your personal health information.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.
You may visit the HSPO’s web site at http://pennstatehershey.org/web/irb/ under participant information for:

- Information about your rights when you are in a research study;
- Information about the Institutional Review Board (IRB), a group of people who review the research to protect your rights; and
- Links to the federal regulations and information about the protection of people who are in research studies. If you do not have access to the internet, copies of these federal regulations are available by calling the HSPO at (717) 531-5687.
Signature and Consent/Permission to be in the Research

Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

**Participant:** By signing this consent form, you indicate that you voluntarily choose to be in this research.

<table>
<thead>
<tr>
<th>Signature of Participant</th>
<th>Date</th>
<th>Time</th>
<th>Printed Name</th>
</tr>
</thead>
</table>

**Person Explaining the Research:** Your signature below means that you have explained the research to the participant and have answered any questions he/she has about the research.

<table>
<thead>
<tr>
<th>Signature of person who explained this research</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
<tbody>
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</table>

**Printed Name**

(Only approved investigators for this research may explain the research and obtain informed consent.)
In addition to the main part of the research study, there is another part of the research. You can be in the main part of the research without agreeing to be in this optional part.

**Optional Storage of Tissue for Future Research**

In the main part of this study, we are collecting blood and urine from you. If you agree, the researchers would like to store leftover samples of your blood and urine. This leftover blood and urine can be used for other research in the future after this study is over.

- These future studies may be helpful in understanding other metabolic side effects of psychiatric treatments.
- It is unlikely that these studies will have a direct benefit to you.
- The results of these tests will not have an effect on your care.
- Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record.
- Sometimes tissue is used for genetic research about diseases that are passed on in families. Even if your samples are used for this kind of research, the results will not be put in your health record.
- It is possible that your blood and urine might be used to develop products or tests that could be patented and licensed. There are no plans to provide financial compensation to you should this occur. If you have any questions, you should contact Dr. Philip Lazarus at (717) 531-5734.

Your leftover samples will be stripped of personal information labeled with a code number.

- These samples will be stored in the locked laboratory and office suits of Dr/ Lazarus at T3427.
- The length of time they will be used is unknown.
- You will be free to change your mind at any time.
- You should contact Dr. Philip Lazarus at (717) 531-5734 and let him know you wish to withdraw your permission for your blood and urine to be used for future research. Any unused blood and urine will be destroyed and not used for future research studies.
You should initial below to indicate what you want regarding the storage of your leftover blood and urine for future research studies.

a. Your samples may be stored and used for future research studies to learn about, prevent, treat or cure metabolic side effects of psychiatric treatments.

   ______ Yes   ______ No

b. Your samples may be stored and used for research about other health problems.

   ______ Yes   ______ No

c. Your samples may be shared with other investigators/groups without any identifying information.

   ______ Yes   ______ No

**Participant:** By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part of the research study.

______________________________
Signature of Participant
Date
Time
Printed Name

**Person Explaining the Research:** Your signature below means that you have explained the optional part of the research to the participant and have answered any questions he/she has about the research.

______________________________
Signature of person who explained this research
Date
Time

__________
Printed Name
Kathryn Erickson-Ridout

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
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<tbody>
<tr>
<td>University of Southern California</td>
<td>B.S.; B.A.</td>
<td>1999-2003</td>
<td>Biochemistry, Chemistry, music studies minor</td>
</tr>
<tr>
<td>Pennsylvania State College of Medicine</td>
<td>M.D.; PhD</td>
<td>2004-2013</td>
<td>Cellular and Molecular Biology</td>
</tr>
</tbody>
</table>

**Honors:** Women in Science and Engineering Award ‘11; PSCOM Class of 1971 Scholarship ‘10

**Memberships:** American Association of Cancer Research, American College of Physicians, American Psychiatric Association, Graduate Women in Science

**Teaching Experience:** Facilitator, PSCOM Problem Based Learning years I and II, 2009-2011

**Positions:** Vice President, Penn State College of Medicine (PSCOM) Student Assembly, 2011-2012; Founder and President, Graduate Women in Science Kappa Rho Chapter, 2010-2011; Internal Advisory Board Member, Women in Science and Engineering, 2010-current; Alternate Member/Member, PSCOM Institutional Review Board, 2010-2011/2011-2012; Community Service Director, Penn State Graduate Student Association (GSA) 2009-2011; GSA Donation Coordinator, Children’s Miracle Network Tisket-a-Tasket Raffle, 2010, 2011; Coordinator, PSCOM Milton S. Hershey School Project Fellowship, 2010-2011; One Warm Coat Drive Coordinator, PSCOM Winter 2009, 2010; Student Doctor, Penn State LionCare Women’s and General Clinic, ongoing; Steering Committee Student Representative, Penn State MD/PhD Program 07/08-07/10; President, PS Physician Scientist Student Interest Group, 07/05-06/06

**Abstracts:**

K.K. Erickson, P. Lazarus. Olanzapine metabolism and the significance of the UGT2B10<sup>48V</sup> and UGT1A4<sup>48V</sup> variants. 25<sup>th</sup> MD/PhD Conference 7/2010. Keystone, CO.

K.K. Erickson, P. Lazarus. Olanzapine metabolism and the significance of the UGT2B10<sup>48V</sup> and UGT1A4<sup>48V</sup> variants, Graduate Women in Science National Meeting 7/2010. State College, PA.

K.K. Erickson, P. Lazarus. Olanzapine metabolism and the significance of the UGT2B10<sup>48V</sup> and UGT1A4<sup>48V</sup> variants. ADA 70<sup>th</sup> Scientific Sessions 6/2010. Orlando, FL.


**Papers:**


**Reviews:**


**Research Support:** 7-08-CST-01 American Diabetes Association, Role: PI, 100% effort, 08/15/09 - 08/15/12; Title: The effects of UDP-glucuronosyltransferase polymorphisms on olanzapine metabolism: implications for obesity and diabetes risk

**Patents:** Lazarus, P. and Erickson, K. Methods Relating to Olanzapine Pharmacogenetics PCT-49918 submitted 3/10/10.